Identification and Applications of Llama-Derived Single Domain Antibodies Binding to Glycoprotein D of Herpes Simplex Virus 2

By

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Abstract

There are currently no protective vaccines or microbicides that can prevent the transmission of herpes simplex virus 2 (HSV-2). The purpose of this thesis was to develop a microbicide based on a llama-derived single domain antibody (VHH) binding to glycoprotein D (gD2) of HSV-2. After immunizing two llamas with gD2 and creating a phage library displaying the VHH repertoire of the immunized llamas, ten unique VHH sequences were identified that bind to gD2. Although many of the VHHs were able to bind gD2 when expressed and purified from *E. coli*, none of the VHHs were able to neutralize the virus *in vitro* or *in vivo*. Only when a gD2-binding VHH called R33 was expressed as a pentamer did it exhibit virus neutralization activity against HSV-2. When R33 was expressed as a fusion protein with the HSV-2 antimicrobial peptide TATC, the *in vitro* antiviral activity of the R33-TATC was increased compared to a non-gD2 binding VHH expressed with TATC and to the TATC peptide alone. Creation of a single domain antibody immunotoxin by expression of R33 with the active domain of exotoxin A from *Pseudomonas aeruginosa* resulted in specific and potent killing of HSV-2 infected cells *in vitro*. Although the isolated VHH that bind to gD2 are unable to neutralize HSV-2 on their own in a monomeric form, they can be used to specifically deliver other effector proteins resulting in potent antiviral activity against HSV-2.

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Chapter One: General Introduction

1. Herpes Simplex Virus-2 (HSV-2)

a. Classification

Almost 100 years have passed since herpes simplex virus (HSV) was formally recognized as the causative agent of the recurrent oral and genital lesions that have plagued mankind for thousands of years[1, 2]. HSV is classified as a member of the *Herpesviridae* family based on four architectural features of the virion: 1) a viral core composed of linear, double stranded DNA; 2) an icosahedral capsid surrounding the core; 3) an amorphous proteinaceous layer called the tegument; and 4) an outer layer composed of a lipid bilayer with viral glycoproteins. The *Herpesviridae* family is divided in to three subfamilies with differing biological characteristics: *Alphaherpesvirinae, Betaherpesvirinae,* and *Gammaherpesvirinae.* HSV is included in the subfamily *Alphaherpesvirinae* due to its broad host range, relatively short reproductive cycle, rapid spread in culture, efficient lysis of infected cells, and ability to establish latent infection in cells of neuronal origins[3].

There are two serotypes of herpes simplex viruses that infect humans, designated HSV-1 and HSV-2. Analysis of complete genomic DNA reveals that they are closely related, and that their 1.5 kilobase pair genomes encode for over 80 proteins[4, 5]. HSV-1 is typically associated with oral lesions, commonly known as cold sores, while HSV-2 primarily infects the genital tract, where it can manifest as genital lesions[6]. Recently it was reported that HSV-2 is actually more closely related to a newly discovered chimpanzee herpesvirus (ChHV). The analysis suggests that HSV-1 and HSV-2 may

have been acquired from different primate species, and that they evolved independently in the separate niches of their human host[7].

b. Epidemiology

Herpes simplex virus 2 (HSV-2) is one of the most common sexually transmitted viruses, with the World Health Organization estimating that approximately 500 million people are infected worldwide[8]. Within the United States, the most recent National Health and Nutrition Examination Survey data indicates that 16% of sexually active 14-49 year olds are infected with HSV-2[9]. Herpes simplex viruses are acquired through mucosal contact, with HSV-1 typically being acquired in childhood through non-sexual contact while HSV-2 is most often sexually transmitted[6]. HSV-1 has historically been categorized as an orofacial infection and HSV-2 as a genital infection, but each virus is capable of infecting either site, and it is becoming apparent that an increasing proportion of genital herpes is caused by HSV-1[10]. HSV-2 infections are distributed unevenly throughout the population in the United States. Women are 45% more likely than men to be HSV-2 positive, and this disparity is most evident in certain racial populations, particularly African-Americans[11]. Increasing age and number of sexual partners are the strongest predictor of HSV-2 infection; seroprevalance rose from 1.4% in the youngest age range (14-19) to 26.1% in the oldest age group surveyed (40-49)[9].

c. Clinical Impact of HSV-2

Initial acquisition of HSV-2 results from unprotected mucosal contact with a person shedding the virus. Replication of the virus first occurs in the epithelial cells at the site of infection, and ultimately spreads to the innervating neurons, resulting in the establishment of a latent infection in the dorsal root ganglion. Primary infection with

HSV-2 was previously thought to always be accompanied by painful lesions at the site of infection, followed by a chronic infection characterized by periodic clinically evident reactivations of the virus[5]. It is now understood that it is common for primary infection and recurrences with HSV-2 to be asymptomatic or for individuals to not recognize their symptoms as being indicative of an HSV-2 infection. In fact, only about 10% of HSV-2 infected individuals are aware of their infection, greatly hindering efforts to prevent the transmission of HSV-2[12]. Additionally, using the control arm of a vaccine study to monitor HSV-2 seroconversion, it was determined that the majority of new HSV-2 infections are acquired without any recognized symptoms[13].

Although viral shedding of HSV-2 in the case of an oral infection is rare[14], shedding from a genital HSV-2 infection is quite common, indicating that HSV-2 is rarely truly latent and the immune system and virus are in almost constant opposition. The most exhaustive study measuring the occurrence of HSV-2 reactivation involved sampling HSV-2 positive patients 4 times a day for 60 days. This study revealed that most HSV-2 reactivations are very short, with half of the reactivations lasting less than 12 hours. Patients experienced episodes at a median rate of 18 episodes per year, but only 19% of these episodes were symptomatic[15]. In terms of the duration of shedding episodes, in subclinical reactivations the virus is present for a mean of 1-5 days, while with symptomatic episodes the virus can be detected for a mean of 1-8 days, although these times may represent multiple overlapping reactivations[16]. A longitudinal study of HSV-2 reactivation rates combined with a model of HSV within-host dynamics indicates that the frequency of virus reactivation and shedding decreases over time, though some individuals still experience high rates of reactivation 4-5 years after primary

infection[17]. The techniques for detecting and predicting viral shedding continue to evolve, and a recent study using mathematical modeling suggests that viral shedding is possibly as frequent as every week, and that daily sampling is insufficient to monitor the frequency of shedding because the very brief virus reactivations are rapidly cleared by peripheral CD8+ T cells[18].

HSV is generally not considered a serious infection in otherwise healthy individuals. The periodic reactivations of the virus are usually resolved quickly but a very small percentage of people can experience more frequent or debilitating reactivations, which can affect quality of life[16]. Additionally, it is recognized that there is often a negative psychological impact of an HSV-2 diagnosis[11]. For those that are immunocompromised, including newborns, HSV can be life threatening. Neonatal acquisition of HSV-2 from the infected mother during birth can lead to dissemination through the central nervous system and encephalitis, and is often fatal even with prompt antiviral treatment[19]. Serious complications from HSV infection are often seen in AIDS patients and other immunocompromised populations[20]. Additionally, an ocular infection with HSV is particularly dangerous due to stromal keratitis and scarring, and HSV is the leading cause of infectious blindness in the US[21].

d. HSV-2 As Cofactor in HIV-1 Epidemic

One of the most serious consequences of an HSV-2 infection is that it is a significant cofactor in HIV transmission[22]. Epidemiological studies initially revealed the link between the two viruses, indicating that prior infection with HSV-2 greatly increases the risk of both transmitting and acquiring HIV-1[23-25]. Strikingly, statistical analysis reveals that 25-35% of HIV-1 infection in Africa may be attributed to HSV-2, and that

HSV-2 facilitates the spread of HIV-1 into otherwise low-risk populations[26]. Biological studies have helped elucidate the mechanisms that underlie the relationship between the two viruses. The disruptions in the epithelial layer that accompany an HSV-2 recurrence can serve as portals of entry and exit for HIV-1[27]. Acute HSV-2 episodes are associated with increased levels of HIV-1 transcription and plasma viral loads[28], and HIV-1 is shed at higher levels in the genital tract during HSV-2 reactivations[29]. Reactivations of HSV-2 in the genital tract, both symptomatic and asymptomatic, induce the infiltration HIV-receptor positive T cells into the genital epithelium[30]. These immune cells remain in the genital tract for months after the herpetic lesion has healed and their persistence is not affected by treatment with acyclovir[31]. HSV-2 has also been shown to have a direct effect on enhancing HIV-1 replication and transcription in co-infected cells[32, 33]. Infection with HSV-2 can induce the expression of HIV-receptors in macrophages, which are one of the most important cell types responsible for HIV transmission[34]. Several clinical trials have demonstrated that suppressive therapy with acyclovir decreases HIV-1 plasma load and occurrence of genital ulcers[35, 36], and can also slow progression of HIV-1 disease[37, 38]. This may be due to the ability of acyclovir to inhibit HIV-1 replication through effects on reverse transcriptase[39, 40]. Treatment of HSV-2 with standard doses of acyclovir unfortunately does not prevent transmission of HIV-1 in discordant couples[41, 42]. The dangerous synergy between the two viruses underscores the urgency in developing novel therapeutics and preventative tools that could potentially prevent the transmission of both HSV-2 and HIV-1.

2. HSV Life Cycle

a. Entry

Although HSV-1 and HSV-2 are distinct serotypes, the basic life cycles of the two viruses are the same. HSV first initiates contact with a susceptible cell through binding of the viral glycoproteins C (gC) and B (gB) to cellular heparan sulfate proteoglycans (HSPGs)[43] or paired immunoglobulin-like receptor α (PILRα)[44]. Once the virus has bound to the cell surface, glycoprotein D (gD) is able to engage one of its receptors: herpesvirus entry mediator A (HveA)[45], 3-O sulfated heparan sulfate[46], or nectin-1[47, 48]. Upon binding, gD undergoes a conformational change that promotes the formation of a fusion complex involving gD, gB, gH, and gL, resulting in fusion of the viral and cellular membranes and delivery of the tegument contents and viral capsid into the cytoplasm of the cell[49]. There is also evidence supporting the role of an endocytic pathway for entry of the virus into the cell, although this appears to be cell-type specific[50]. Several of these entry steps are susceptible to blockage by small molecules or antibodies, thereby preventing entry of the virus into the cell. While glycoproteins gB, gH, and gL are conserved among all herpes viruses, gD2 is also essential for entry of HSV-2[51]. Neutralizing antibodies against these glycoproteins demonstrate that the targeting of essential glycoproteins may be a feasible point of intervention to prevent entry of the virus in to the cell.

b. Viral gene expression

Once the virus has successfully entered the cell, viral proteins immediately begin reshaping cellular processes to allow for immune evasion, replication of cellular DNA, and assembly and egress of the virus. Tegument proteins released into the cytoplasm

upon fusion of the viral and cellular membrane have diverse targets within the cell, reviewed by Melchjorsen et al. (2009)[52]. One of the most important functions of the tegument proteins is to halt the cascade of signaling events within the cell that is initiated upon recognition of the virus by the innate immune system, such as the Nuclear Factor kappa B (NF-kB), IFN Regulatory Factors (IRFs) and Mitogen-Activated Protein Kinase (MAPK) pathways[52]. The tegument protein VP22 is responsible for the distribution and subcellular localization of various tegument proteins that are critical in subverting the innate defenses of the cell and allowing the establishment of an infection[53]. The viral capsid is transported to the nucleus using the cellular microtubule network[54, 55], and upon interaction of the capsid with a nuclear pore, the viral DNA is released into the nucleus[56].

Transcription and replication of viral DNA occurs using a combination of virally encoded and host encoded proteins. Viral gene transcription is a tightly regulated cascade in which gene products from the preceding set of genes control expression of the following set of genes. The tegument protein VP16, which travels to the nucleus independently of the capsid upon entry to the nucleus[57, 58], is critical in assembling the complex of host proteins that promote the transcription of the viral immediate-early genes, or α genes[59]. The immediate-early genes encode the proteins that are responsible for initiation of transcription of the early, or β genes. β gene transcription is required for the expression of β gene products that are necessary for amplification of the viral genome, and also for stimulating the transcription of late genes, or γ genes. γ gene products comprise the virion structural components and are responsible for the assembly

of the newly replicated viral genome with the viral proteins necessary for infection of the next susceptible cell[5].

c. Capsid assembly and egress

Once the viral DNA has been replicated and the capsid structural gene products have been expressed, encapsidation of the viral genome occurs. Partially assembled capsid proteins with the internal scaffold protein make their way from the cytoplasm to the nucleus, where further cleavage and assembly events result in an immature capsid complex ready for packaging of the viral DNA[60, 61]. In this process, the newly replicated and concatamerized viral DNA is packaged into pre-assembled capsids and cleaved into a single viral genome unit, displacing the scaffold and with enough force that the packaged DNA is of crystalline-liquid density[62].

Upon successful encapsidation of the viral DNA, the virus embarks upon the process of egress from the cell. The complex rearrangement of cellular membranes and stages of viral de-envelopment and envelopment is exhaustively reviewed by Johnson and Baines (2011)[63]. To summarize, the viral capsid starts the process by budding in to the perinuclear space from the inner nuclear membrane, a step referred to as primary envelopment. Many of the viral glycoproteins involved in entry of the virus into the cell are involved in this budding step, as well as in the fusion step of the enveloped capsid with the outer nuclear membrane, known as de-envelopment. This releases the capsid in to the cytoplasm without any envelope, but still surrounded by the tegument proteins it has acquired from the nucleus, perinuclear space, and in the cytoplasm. The tegument-capsid complex then undergoes secondary envelopment into cytoplasmic membranes such as the Golgi, *trans* Golgi network, and endosomes. These cytoplasmic membranes

contain the required viral glycoproteins necessary for viral entry, so that upon transport of these vesicles to the cell surface and fusion with the cellular membrane, the released enveloped virions are mature and infectious[63].

3. Latency

One of the most distinctive features of members of the *Alphaherpesvirinae* is their ability to latently infect neuronal cells and establish a lifelong infection. In epithelial cells at the site of infection, HSV undergoes the lytic cycle of replication described previously, but the virus is also able to undergo a different program of viral gene expression in neuronal cells to achieve a latent state with intermittent periods of reactivation[5]. Viral entry occurs at the axonal termini of sensory neurons in the same manner as in epithelial cells, with nectin-1 being the primary receptor that gD2 engages on neurons to initiate fusion[64]. The viral capsid is then carried to the nucleus in the cell body by retrograde axonal transport, a process reviewed by Smith (2012)[65]. Upon entry into the nucleus, the viral DNA either begins replication for a productive infection and release of progeny virus, or circularizes and becomes associated with nucleosomes for a latent infection[66]. The determining factor for which pathway the virus takes is dependent on the presence of VP16 in the nucleus. Even though VP16 is separated from the capsid after entry in to the neuron, it appears that in some instances a small amount of VP16 may remain associated with the capsid and enter the nucleus upon infection of a neuron. If this occurs, the virus progresses along the lytic cycle of replication. If VP16 is not present in the nucleus, however, latency is initiated. Additionally, other cellular nuclear factors are thought to play a role in suppressing activation of transcription and viral gene expression[67].

In the latent state, production of infectious virus does not occur and no viral genes are transcribed except for the latency-associated transcript (LAT), an unusually stable RNA molecule that is cleaved into smaller, similarly stable RNA species. The literature regarding the function of LATs is vast and contradictory, but common themes have emerged indicating their ability to maintain latency by mechanisms such as repressing viral gene transcription and inhibiting neuronal apoptosis[66, 67]. The factors regulating reactivation of the virus are not completely understood, but involve a decrease in the transcription of the LATs and epigenetic reorganization[68] of the viral genome to allow transcription of α, β, and γ genes[67]. In humans, reactivation can be triggered by injury or stimulation of cells innervated by latently infected neurons. In the case of reactivation, the viral DNA undergoes transcription and replication as it does during a lytic infection. This results in newly formed viral capsids traveling to the distal axon by antereograde transport and infectious virus is released at or near the original site of infection[65].

4. Immunity to HSV-2

As a result of the reservoir of latently infected neurons, an HSV infection persists through the life of the host. Infection with HSV does, however, induce a robust cellular and humoral immune response in immunocompetent hosts. If the virus can get past the nonspecific antiviral defenses of the vagina, such as low pH, complement, and antimicrobial peptides[69, 70], it is able to enter the susceptible cells of the genital epithelium. Upon infection, epithelial cells are able to recognize HSV through pattern recognition receptors (PRRs), which detect conserved regions of the virus, called pathogen-associated molecular patterns (PAMPs). The primary PAMPs detected within

infected epithelial cells are viral nucleic acids, particularly the unmethylated CpG motifs[71], which are detected by the PRR Toll-like receptor 9 (TLR9), and double stranded RNA viral replication intermediates, which are detected by TLR3[72]. This recognition stimulates the production of cytokines and release of interferons, which serve to alert other cells of the innate and adaptive immune system to the viral infection and ultimately limit the extent of viral infection in the periphery. The first wave of immune cells recruited to the site of infection includes monocytes, neutrophils, dendritic cells (DCs), and natural kills (NK) cells. These cell types work together to induce apoptosis of infected epithelial cells, phagocytose dying cells, and present viral antigen to cells of the adaptive immune response[73]. The first line of the adaptive immune response against HSV-2 in the vagina is primarily achieved through the induction of a humoral immune response, and more specifically an IgG response[74]. Although B-cells are able to produce neutralizing antibodies directed at viral proteins, it appears their most important function in controlling HSV replication is to function as antigen presenting cells to stimulate activation of T cells[75].

T cells play the most critical role in the adaptive immune response to HSV infection. T cell depletion in HSV-2 infected mice leads to impaired resolution of infection[76] and clearance of the virus from mucosal tissues depends on infiltration of HSV-specific cytotoxic T lymphocytes[77]. CD4 + T cells typically arrive at the infected area before CD8 + T cells and secrete cytokines, including interferon-γ, tumor necrosis factor-α, interleukin (IL)-2 and IL-4, upon encountering viral antigen which serve to abate the viral infection through non-cytolytic mechanisms[78]. $CD8^+$ T cells play a critical role in controlling HSV infection by killing infected epithelial cells and maintaining latency in

infected neurons[79, 80]. When $CD8^+$ T cells recognize viral antigens presented in the MHC class I complexes of target cells, they exert their cytotoxic activity by secretion of interferon-γ, TNF-α, perforin, and granzymes. TNF-α, perforin, and granzymes all induce infected cells to undergo apoptosis, while interferon-γ enhances MHC class I presentation of viral peptides, arrests the cell cycle, and inhibits viral replication in infected cells. Interferon-γ also promotes the development of naïve T cells towards a T_H1 immune response, which has shown to be the predominant protective immune response to HSV-2[81]. More specifically, it is known that infection of mice with an attenuated HSV-2 strain induces a protective T_H1 immune response against subsequent wild type challenge[82-84].

These cytolytic antiviral mechanisms are sufficient to control viral replication in mitogenic cells of the periphery since they can be replaced. Neurons, however, are nonmitogenic and a different strategy is required to maintain HSV in a latent state and prevent reactivation. It appears that very low levels of viral gene expression maintains a population of virus-specific CD8+ T cells, predominantly recognizing gB[85], that are continually in contact with the infected neurons[86]. Apoptosis is rarely induced in neurons, despite CD8+ T cells releasing a similar combination of effector molecules such as perforin, granzymes, and interferon-γ, although the exact mechanisms regulating the lack of cytotoxicity is not understood[87, 88]. Interferon-γ released by CD8+ T cells is able to induce expression of MHC class I receptors in neurons that otherwise do not express it[89], and it is also able to regulate global transcription to decrease viral gene expression[90]. It is apparent that a combination of viral mechanisms that actively prevent apoptosis as well as a differential responses to traditional cytolytic factors in

neurons are responsible for keeping latently infection neurons from undergoing apoptosis[80, 91].

There is considerable variability among individuals in terms of the efficacy of the immune response in controlling HSV-2 infection and the frequent reactivations of the virus. Many people become infected with HSV-2 and never experience symptoms or a reactivation, while others experience multiple symptomatic viral reactivations each year[92]. Mathematical modeling predicts that it is the level of the mucosal immune response rather than virological factors that is responsible for controlling the duration and severity of viral shedding[18]. Specific genotypes in humans have been associated with the severity of the infection and susceptibility to encephalitis[93, 94]. Additionally, it has been reported that people who experience symptomatic infection have a distinct T cell response compared to those with an asymptomatic infection, and that reactivity to a specific set of HSV epitopes may be a critical determining factor for whether an infection is symptomatic or asymptomatic[95, 96]. The immune system, however, is not all to blame for incompletely controlling an HSV-2 infection. In addition to the ability of HSV-2 to undergo a different program of viral replication in neurons to establish latency, HSV also encodes multiple proteins that serve to actively inhibit the immune system, which is explored in detail by Roizman et al[5].

5. HSV Treatments and Prevention

Unfortunately, HSV-2 treatment and prevention methods have not significantly advanced with the accumulation of the vast quantity of research dedicated to understanding HSV. Condoms provide some protection against sexual transmission, but

they do not entirely cover the susceptible genital region where transmission can occur[97, 98]. Treatment with DNA nucleoside analogs such as acyclovir, ganciclovir, and famciclovir are effective at reducing frequency of clinical reactivation and in preventing transmission of HSV-2 to seronegative partners[99]. The development of a therapeutic vaccine that could be used in HSV-2 positive individuals to alleviate symptoms and prevent viral shedding is an active area of research, although they have only been marginally successful in clinical trials[100]. Because infection with HSV-2 is lifelong and the virus cannot be eliminated from the latently infected neurons by the immune system, prevention of infection from occurring is of key importance. Two ways that this could be accomplished is: a) through a vaccine that elicits sterilizing immunity and b) development of a topically applied microbicide to prevent transmission.

a. HSV-2 Prophylactic Vaccines

Starting in the 1980s, it appeared progress was being made in the development of an HSV-2 vaccine using protein-based vaccines containing glycoproteins found on the surface of the virion[101]. One of the most promising vaccine candidates was recombinant gD2; gD2 had been well characterized, and a great deal of information was known about its structure, function, and the location of immunogenic epitopes within the protein[102, 103]. Furthermore, it was determined that antibodies directed against this glycoprotein can prevent infection *in vitro*[104, 105]. Its ability to stimulate production of neutralizing antibodies in animals (mice and guinea pigs) was demonstrated many times over, and as a result, it became a top candidate for vaccine development[106-108]. Several large-scale clinical trials in humans were conducted by pharmaceutical companies including Merck, Chiron, and GlaxoSmithKline, and vaccines using gD2

injected with various adjuvants and immunization schedules were evaluated. All of the trials failed to demonstrate significant protection against acquisition of the virus[109-112]. While the vaccines were generally able to induce serum antibody responses against the virus, an important component of HSV-2 immunity, it became apparent that they were unsuccessful in stimulating a protective T cell response and sufficient antibody concentration at the site of infection: the genital tract[113]. The trials and tribulations of HSV-2 vaccine development over the past 30 years are documented at length in several recent reviews[100, 101, 114].

Vaccines against STIs have been notoriously difficult to develop, and to date the only STI that has been successfully targeted with a vaccine is human papilloma virus (HPV)[115]. The genital tract is distinct from other regions of the body due to the lack of established lymphoid follicles and the need for immunological tolerance against sperm and the developing fetus, resulting in a general dampening of the immune response[70, 116]. Through animal studies, it has become clear that a successful vaccine against HSV-2 must elicit a local immune response in the genital tract, with both cellular and humoral components[116, 117]. HSV-2 vaccine research is still a robust area of investigation despite the setbacks of the clinical trials, and the information learned from the unsuccessful trials has been guiding exciting ongoing research. Instead of the traditional subunit vaccine approach, many groups are now focusing on diverse strategies such as vaccination with HSV DNA, specific immunogenic peptides, and attenuated or replication deficient HSV[118]. Another recent direction is to use a mucosal vaccination approach, in which HSV-2 antigens are introduced at the site of infection to draw and maintain the appropriate immune cells where they are needed to prevent infection[119].

b. HSV-2 Microbicides

As an alternative to a prophylactic vaccine against HSV-2, researchers have been earnestly pursuing the development of a microbicide to prevent the sexual transmission of HSV-2. Microbicides are substances that can be applied vaginally or rectally to prevent acquisition of an STI. Ideally, a microbicide is nontoxic, is not immunogenic, doesn't disrupt the commensal flora of the genital tract, is easy to apply, and transparent to users. With these characteristics, it may be possible to develop a microbicide that a woman could consistently and covertly use to protect herself from an STI. There has been a great deal of research identifying molecules that have excellent antiviral activity against the virus *in vitro* and potential for use as a HSV-2 microbicide. A non-exhaustive list of such microbicide candidates includes antimicrobial peptides[120-123], DNA aptamers[124], dendrimers[125, 126], and antiviral drugs such as tenofivir[127]. Additionally, antibodies have excellent potential to meet the criteria of an acceptable microbicide given their safety profile and neutralization and binding capabilities. For example, microbicide studies in several animal models have demonstrated that antibodies binding to gD2 passively administered to the vagina were effective at protecting against genital HSV-2 infection[128, 129]. Microbicides with efficacy against both HIV and HSV-2 are particularly attractive, especially given the overlapping epidemics and synergy between the two viruses[130]. Only a few microbicide candidates, generally originally aimed at preventing HIV-1, have been evaluated in human clinical trials for efficacy in preventing HSV-2 transmission (including Carragaurd, cellulose sulfate, PRO2000, Nonoxynol-9, tenofovir)[130]. Only tenofovir, an HIV reverse transcriptase inhibitor formulated as a vaginal gel, showed efficacy in preventing HSV-2 transmission in humans[131, 132], a

result that is currently being followed up and investigated. If confirmed, in addition to the interesting finding that an HIV drug has efficacy against HSV, it also demonstrates that with the correct antiviral candidate, the strategy of vaginal application of a microbicide can be effective at preventing HSV-2 transmission. This is encouraging news for the numerous promising HSV-2 microbicides that are currently in development.

6. Purpose of thesis work

The purpose of this thesis work was to develop a unique HSV-2 microbicide candidate that addresses the requirements for an ideal microbicide. The proposed strategy was to utilize the advantageous properties of llama-derived single domain antibodies (VHH) to develop a potent and robust microbicide expressed *in situ* by a commensal bacterium to achieve sustained delivery. Immunization of llamas with the antigen of interest (gD2) could be used to elicit and ultimately identify the genes encoding a neutralizing VHH. Many of the components underlying this strategy have previously been validated, and are discussed further in the following chapters: 1) VHH have been used to neutralize other viral targets at biologically relevant concentrations[133]; 2) the viral protein, gD2, has been studied at length and is known to be an efficient target for neutralizing the virus both *in vitro* and *in vivo[129, 134]*; and 3) commensal bacteria have been used to express a therapeutic VHH for sustained *in vivo* delivery[135]. The original research plan was carried out and anti-gD2 VHH were identified and evaluated as microbicide candidates, and in addition, further applications of these anti-gD2 VHH, designed to enhance their therapeutic efficacy were explored.

Chapter Two: Development of a Llama Single Domain Antibody Targeting Glycoprotein D of Herpes Simplex Virus 2 as a Microbicide

Introduction

HSV-2 is one of the most prevalent sexually transmitted infections (STIs) in the world, and recent estimates indicate that roughly 16% of people ages 15-49 worldwide are infected[8]. There has been great interest in the development of a prophylactic vaccine to prevent HSV-2 infection over the past several decades, but unfortunately, an effective one has yet to be developed[136]. As a result, there is renewed interest in an alternative strategy to prevent transmission, including the development of an effective microbicide.

A microbicide is a substance that can be applied to mucosal surfaces, including the vagina and rectum, to prevent infection with an STI. There are various avenues being pursued to develop a successful microbicide against HSV-2, including vaginal delivery of antiviral drugs, antibody-based strategies, and small-interfering RNAs[130]. Antibody-based strategies have particular promise considering their safety profile and specificity for viral targets. Additionally, it has already been demonstrated that vaginally applied monoclonal antibodies[134, 137] and single chain antibody variable fragments (scFv)[129] directed against gD2 protect against HSV-2 infection in animal models. The issue of how to vaginally deliver a neutralizing antibody against gD2 without the direct application of the antibody immediately prior to sexual intercourse has yet to be resolved, however. Furthermore, the current methods of production of monoclonal antibodies and

scFvs can be cost-prohibitive to scale up, as antibodies are complex molecules with multiple protein chains that are not easily purified and assembled[138].

The isolation of an antibody fragment that neutralizes HSV-2 and is structurally simple enough to be produced by the native bacterial flora of the vagina would solve both the production and delivery challenges of the antibody-based HSV-2 microbicide strategy. Members of the *Camelid* family (camels, alpacas, and llamas), naturally produce antibodies that are devoid of light chains, so that the antigen binding region is solely contained in the variable region of the heavy chain, referred to as VHH (Figure 2.1)[139]. These VHH domains retain the potent binding capacity of full-length antibodies, are stable under a wide range of temperature and pH conditions, and are not immunogenic[140]. Another attractive characteristic of VHH antibodies is that they are small enough to be secreted by *Lactobacilli*, an ideal organism for delivery of VHH antibodies because they are a major component of the vaginal flora and because systems for expression of heterologous proteins have been developed for these bacteria. A similar approach used *Lactobacilli* secreting a VHH against rotavirus in the gastrointestinal tract to prevent diarrhea[135], and *Lactobacilli* have also been engineered to protect themselves from phage lysis through production of an anti-Lactococcus phage VHH[141]. While transformed *Lactobacilli* are ultimately the likely bacterial species that will be used to protect women against HSV-2, initial *in vitro* and mouse *in vivo* studies to test the efficacy of a VHH microbicide will use *Streptococcus gordonii*, a human oral commensal bacteria, which, unlike lactobacilli, is known to colonize the vagina of mice[142].

It is our hypothesis that a VHH that binds to gD2 of HSV-2 and is secreted by a commensal bacterium could serve as an effective microbicide against sexual transmission of HSV-2. Using phage display technology, we were able to isolate several VHH from immunized llamas that specifically bound to gD2 as measured by ELISA analysis. These gD2-specific VHH were then purified and evaluated for their ability to neutralize virus, using both *in vitro* and *in vivo* assays. Additionally, multivalent forms of the VHH were generated by constructing bivalent and pentavalent VHH, and these were also tested for antiviral activity. While none of the monomeric or bivalent VHH demonstrated any significant virus neutralizing activity *in vitro* or *in vivo*, pentavalent VHH did demonstrate modest *in vitro* HSV-2 neutralization activity. Although the monomeric properties of VHH make them attractive as microbicide candidates, it appears that a pentameric form is required for neutralizing activity for the anti-gD2 VHH that were identified.

Materials and Methods

Expression and Purification of Recombinant His-gD2 from Pichia pastoris

Using genomic DNA from HSV-2 (strain 186) as a template, DNA encoding amino acids 1-314 of gD2 (ectodomain) were amplified from the viral genome using primers (forward) CCCGAATTCACCATGAAATACGCCTTAGCAGACCCCTCG and (reverse) ATTGCGGCCGCGTTAatggtgatggtgatggtgCGGGTTGCTGGGGGC, which also added a His tag to the C-terminus. The gD2 sequence was cloned into the expression vector pPIC9 and transformed into *Pichia pastoris* by electroporation. A mid-scale culture (~30 mL) of Buffered Glycerol-complex Media (BMGY) was inoculated with 500 µL of a gD2/*P. pastoris* glycerol stock and grown at 30°C shaking at 225 rpm for ~48 hrs, until the cultures reaches an OD_{600} of 2-6. The culture was then diluted in 700 mL of BMGY media and grown in a 2 L-baffled flask at 30°C with shaking at 225 rpm until the OD_{600} reached 50. Cells were harvested in sterile centrifuge bottles at 2500 g for 20 minutes at room temperature (RT). To induce expression, the cell pellet was resuspended in 200 mL of Buffered Methanol-complex Media (BMMY) and grown for 48 hrs at 30°C with shaking at 225 rpm. Cells were harvested by centrifugation at 1500-3000 g and supernatant was collected; 2 mL Ni-NTA Superflow Resin (QIAGEN, Valencia, CA) equilibrated in PBS was added per 45 mL supernatant and rocked overnight at 4°C. Resin was collected by centrifugation and washed three times with 50 mL PBS. gD2 was then eluted from the resin by adding 4 x 1 mL elution buffer (250mM imidazole in PBS). Eluted gD2 was filtered through a 0.22-micron filter and dialyzed overnight against PBS. Protein concentration was measured using a Bradford Assay (BioRad, Hercules, CA).

Detection of gD2 purified from Pichia pastoris

NUNC Maxisorp enzyme-linked immunosorbent assay (ELISA) plates (Thermo Fisher Scientific Inc., Waltham, MA) were coated with 0.5 µg purified protein per well overnight at 4°C. Plate was blocked with 2% BSA in PBS for 30 minutes at RT. Primary antibodies, including R45 (rabbit polyclonal, gift from R. Eisenberg and G. Cohen, University of Pennsylvania, Philadelphia), HSV8 (human monoclonal, gift from L. Zeitlin, Mapp BioPharmaceuticals, San Diego, CA), DL6 (mouse monoclonal, (Santa Cruz Biotechnology, Dallas, TX), and anti-His (mouse monoclonal, Sigma-Aldrich, St. Louis, MO), were diluted in PBS-Tween 0.2% (PBS-T) and added to appropriate wells in duplicate for 1 hr at RT. Wells were washed 5 x with 200 µL PBS-T per well and appropriate horse-radish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) was diluted in PBS-T and added to wells for 1 hr at RT. Wells were washed 5 x with 200 µL PBS-T per well and developed using ABTS® ELISA HRP Substrate (KPL, Gaithersburg, MD). The plate was read at 405 nm using a BioTek Synergy HT Plate Reader (Winooski, VT).

Llama Immunizations

The immunization of two llamas, Paquito and Rayo, was performed by Triple J Farms in Bellingham, WA (Protocol #110, approved by Triple J Farms IACUC, USDA registered #91-R-0054). The immunizations occurred on days 0, 21, 42, 63, and 280. Each llama was immunized with 0.5 mg of gD2 per injection, mixed with complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for subsequent injections. Prior to the first immunization and following each immunization, ~20 mL of

serum was collected to monitor for the presence of anti-gD2 antibody. After the fourth and fifth immunizations, 500 mL of blood was taken from each animal and peripheral blood mononuclear cells (PBMCs) were purified using a Ficoll-Paque Plus gradient (GE Healthcare Life Sciences, Piscataway, NJ). PBMCs were aliquoted and frozen at -80C until further use.

Llama Serum ELISA

NUNC Maxisorp ELISA plates (Thermo Fisher Scientific Inc., Waltham, MA) were coated with 100 µl of gD2 at 10 µg/mL and incubated overnight (ON) at 4°C. The plate was blocked with 2% BSA in PBS for 30 minutes at RT. Freshly thawed serum samples were diluted 1:10,000 in PBS and added in duplicate to wells for 1 hr at RT. Wells were washed 5 x 200 µl PBS-T per well and HRP-conjugated anti-llama secondary antibody (Bethyl Laboratories, Inc) was diluted 1:10,000 in PBS-T and 100µL was added to wells for 1 hr at RT. Wells were washed 5 x with 200 µl PBS-T per well and developed with 200 µL ABTS® ELISA HRP Substrate (KPL, Gaithersburg, MD). The plate was read at 405 nm using a BioTek Synergy HT Plate Reader (Winooski, VT).

Llama Serum Neutralization Assay

Vero cells were plated in Falcon 12-well trays (Thermo Fisher Scientific Inc., Waltham, MA) at $4x10^6$ cells per tray and incubated ON at 37°C. Llama serum samples were heat inactivated at 56°C for 60 minutes and serial two-fold dilutions were made in Dulbecco's Modified Eagle Medium (DMEM) / 2% fetal bovine serum (FBS). Approximately 5000 plaque forming units (pfu) / mL of HSV-2 G (ATCC VR-734, Manassas, VA) was added

to each dilution and all dilutions were incubated at 37°C for 1 hr. Media was removed from the Vero cells and the serum dilutions with virus were added in duplicate to cells for 1 hr at 37°C, with gentle shaking every ten minutes to distribute the liquid over the cells. The inoculum was then removed from cells and cells were overlaid with 2 mL 2% methylcellulose overlay/5% FBS in DMEM (Cellgro, Manassas, VA). Trays were incubated for 3 days at 37°C, stained with crystal violet, and plaques were counted.

Amplification of VHH Regions and Construction of T7 Phage Display Library

Using PBMCs that were isolated following the fourth (Rayo) or fifth (Paquito) immunization, RNA was extracted using an RNeasy Mini Kit (QIAGEN, Valencia, CA) and reverse transcribed into DNA (SuperScript II Reverse Transcriptase, Invitrogen, Carlsbad, CA). Nested PCR was performed to amplify the VHH regions from the genomic DNA using primers that bind to the conserved regions flanking the VHH genes. The first round of PCR was performed with primers as previously published[143] while the second round of primers introduced the appropriate restriction sites for ligation into the phage genome. The VHH band of ~450 base pairs was gel extracted and ligated into pre-digested T7 phage vector arms as described in the manufacturer's handbook (Novagen Inc., Madison, WI). The ligation reaction was packaged into the phage according to the manufacturer's protocol and titered to determine the diversity of the packaged library prior to amplification. After amplification, the library was aliquoted and stored at -80°C until further use. VHH expressed on the phage surface are referred to as VHH-phage.

Biopanning of VHH/T7 Library Against gD2

For the first round of biopanning, 10^9 pfu from the phage library was added to a well coated with 0.5 μg gD2 and incubated at room temperature for 1 hr. Wells were then washed 10 times with shaking for 1 minute with tris-buffered saline (TBS) with 0.05% Tween (TBS-T) and 10 times with TBS. Bound phage were eluted using 200 μl of 1% SDS in TBS incubated on wells for 1 hr at room temperature. A sample of the eluted phage was used to titer the amount of phage present, and the remaining eluted phage were added to 50 mL of *E. coli* strain BLT5403 grown in LB/Amp at OD_{600} 0.5 and shaken at 37°C until lysis occurred. This phage lysate was titered and used as the input for the next round of biopanning, which was carried out using the same procedure. Additional rounds of biopanning were performed against gD2 and individual plaques from the phage elution after the second (Paquito) or sixth (Rayo) round of biopanning were picked, amplified, and sequenced.

Antibody Capture Biopanning

Antibody capture biopanning was performed based on a previously published protocol[144]. It was carried out as described above, except that the ELISA wells were first coated with 0.5 ug per well of the non-neutralizing gD2 capture antibody, DL6 (Santa Cruz Biotechnology, Dallas, TX). After this coating step, gD2 was added and then the biopanning protocol proceeded as described in the previous section.

VHH-phage ELISA

An ELISA was performed to determine if individual VHH-phage clones could bind to gD2. NUNC Maxisorp ELISA plates (Thermo Fisher Scientific Inc., Waltham, MA) were coated with 0.5 µg gD2 per well and incubated ON at 4°C. The plate was blocked for 1 hr with 2% BSA in PBS, and then 10^9 pfu of each phage clone was added in duplicate and incubated at RT for 1 hr. After removing phage, the plate was washed 5 x 200 µL PBS-T per well. Anti-T7 tail fiber monoclonal antibody (GE Healthcare Life Sciences, Piscataway, NJ) was diluted to 1:1000 and added in 100µL to each well for 1 hr at RT. After washing the plate 5 x 200 µL PBS-T per well, HRP-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA) was added at 1:3000 in 100µL and incubated at RT for 1 hr. After a final wash of 5 x 200 µL PBS-T per well, 200 µL of ABTS® ELISA HRP Substrate (KPL, Gaithersburg, MD) was added. The plate was read at 405 nm using a BioTek Synergy HT Plate Reader (Winooski, VT).

Cloning and Expression of VHH in E. coli

VHH sequences were amplified from phage by PCR amplification using the primers that introduced EcoRI and XhoI restriction sites for cloning in to pET-47b (Novagen Inc., Madison, WI). Additional primer sets were used to amplify VHH and insert a second VHH sequence with a GS linker between them to make a bivalent VHH construct. The monovalent and bivalent VHH constructs were transformed in to *E. coli* BL21 DE3 competent cells (New England Biolabs, Ipswich, MA). Two methods of expression and purification were utilized depending on the solubility of the VHH protein.

1) *Osmotic shock*: For the VHH that were soluble (all VHH derived from Rayo, indicated by R##), an osmotic shock protocol was utilized to purify protein from the periplasmic space, as described by Graef et al[145]. Briefly, an ON 30 mL mid-scale culture was diluted in 450 mL Terrific Broth and grown at 25°C for 3 hrs. Cells were induced at 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Lab Scientific, Inc, Highlands, NJ) and grown for an additional 3 hrs at 25°C. After centrifugation, the cell pellet was lysed in Tris-sucrose buffer with lysozyme. Contents of periplasmic space were separated from cellular debris by centrifugation and Ni-NTA Agarose (QIAGEN, Valencia, CA) was added to the supernatant ON with rocking at 4°C. Agarose was collected by centrifugation and washed, and protein was eluted by addition of 3 mL elution buffer.

2) *Insoluble protein purification*: For those VHH that were insoluble (all VHH derived from Paquito, indicated by P##), an ON 10 mL culture was diluted into 750 mL of Luria Broth (LB) / kanamycin (kan) and grown until OD_{600} 0.6-0.8. After induction with IPTG at 1 mM for 3hrs at 37°C, cells were harvested by centrifugation at 3500 g for 30 minutes, resuspended in 10 mL lysis buffer (6 M Guanidine hydochloride, 0.1 M NaH_2PO_4, 0.01 M Tris base, 0.01 M imidazole, pH 8) and frozen at -80°C for at least 30 minutes. Upon thawing, the volume of the lysate was brought to 30 mL with lysis buffer, incubated with rocking at RT for at least 30 minutes, and then centrifuged at 14000 rpm for 30 minutes. After the pellet was discarded, Ni-NTA Agarose (QIAGEN, Valencia, CA) was added to lysate and rocked at RT for 1 hr or ON at 4°C. Beads were washed twice with 7 mL Wash Buffer 1 (8 M urea, 0.1 M NaH_2PO_4, 0.15 M NaCl, 0.02 M imidazole,

pH 8) and then washed with ~50 mL (7 x 7 mL) Wash Buffer 2 (0.05 M NaH_2PO_4, 0. 5 M NaCl, 0.02 M imidazole, pH 8). To elute VHH from beads, 4 x 1 mL Elution Buffer (0.05 M NaH_2PO_4, 0.5 M NaCl, 0.25 M imidazole, pH 8) was added for 1 hr at RT.

For both protein purification methods, eluted VHH were dialyzed against PBS with 1 mM dithiothreitol (DTT) with at least 4 buffer changes. VHH were concentrated with Amicon Ultra-15 Centrifugal Filter Unit (EMD Millipore, Billerica, MA), centrifuged at 16000 g for 10 minutes to remove precipitated protein, and protein concentration was measured by Bradford assay (BioRad, Hercules, CA).

Cloning and Expression of Pentavalent VHH

To create a pentavalent VHH, the pVT2 plasmid was obtained from C. Roger Mackenzie (National Research Council Canada, Ottawa, Ontario, Canada). This plasmid allows for cloning of VHH as a N-terminal fusion protein with the verotoxin B subunit, resulting in self-assembly in to a pentamer[146]. R33 expressed as a pentamer will be referred to as NR33. The pentavalent R33/pVT2 construct was transformed into competent BL21 DE3 cells (New England Biolabs, Ipswich, MA) and expressed and purified as previously described[147]. Purified NR33 was run through a Superdex200 column to verify self-assembly.

Coomassie and Western Blot of Purified gD2 and VHH

To verify the size of purified protein, approximately 800 ng of gD2 or each VHH sample was run on a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)

gel for Commassie staining and approximately 200 ng of each sample was run for a Western blot. For Western blotting, samples were transferred to polyvinyl difluoride (PVDF) membrane by a semi-dry transfer system (Biorad Trans-Blot SD Semi-Dry Transfer Cell, Hercules, CA) and detection was performed using standard techniques. Briefly, PVDF membrane with transferred protein was blocked with 5% milk for 1 hr at RT or ON at 4°C. Primary antibody was diluted in PBS-T and incubated on blot for 1 hr at RT with rocking. Blot was washed 4 x 10 minutes with PBS-T, and alkaline-phosphatase (AP)-conjugated secondary antibody (Jackson ImmunoResearch Inc., West Grove, PA) was diluted in PBS-T and added to blot. After a final wash of 4 x 10 minutes in PBS-T, NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) detection reagents were added until bands were visualized.

ELISA to Validate VHH Binding to gD2

An ELISA was performed to determine if purified VHH binds to gD2. Wells of NUNC Maxisorp ELISA plates (Thermo Fisher Scientific Inc., Waltham, MA) were coated with various dilutions of VHH made in PBS and incubated ON at 4°C. Purified gD2 diluted in PBS-T was added to wells for 1 hr at RT. Wells were washed 4 x 200 µL PBS-T and the anti-gD antibody DL6 (Santa Cruz Biotechnology, Dallas, TX) diluted in PBS-T was added to detect gD2 binding by VHH. After a 1 hr incubation at RT, wells were washed again 5 x 200 µL PBS-T and an anti-mouse secondary antibody conjugated to HRP (Jackson ImmunoResearch, West Grove, PA) was added. After a final wash with PBS-T 4 x 200 µL, 200 µL ABTS® ELISA HRP Substrate (KPL, Gaithersburg, MD) was

added. The plate was read at 405 nm using a BioTek Synergy HT Plate Reader (Winooski, VT).

Flow Cytometry to Validate VHH Binding to Surface Expressed-gD2

Z4/6 cells (gift from D. Johnson, Oregon Health and Science University) are a derivative of L cells that stably express gD2 at the cell surface[148]. Nearly confluent cells were trypsinized, washed once with PBS, and resuspended at 0.5 x 10^6 cells/mL. 500 µL of cells were aliquoted, centrifuged at 500 g for 5 minutes, and resuspended with 1 mL 1% BSA/PBS and incubated at 37°C for 30 minutes for blocking. Samples were centrifuged at 500 g for 5 minutes, resuspended in VHH or DL6 antibody (Santa Cruz Biotechnology, Dallas, TX) diluted in 1% BSA/PBS, and incubated for 1 hour at 4°C. Cells were washed twice with 2 mL PBS and resuspended in appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted in 1% BSA/PBS for 30 minutes at 4°C, followed by a final wash with 2 mL PBS. Samples were run on a Becton-Dickinson FACSCalibur Cytometer and data was analyzed using FloJo (Tree Star Inc., Ashland, OR).

Expression of VHH by S. gordonii and Validation by ELISA and Flow Cytometry

VHH sequences were amplified using primers to introduce the appropriate restriction sites for cloning into the pLEX plasmid, as well as to introduce an N-terminal His tag and C-terminal myc tag. VHH expressed at the surface on *S. gordonii* will be referred to as VHH/Sg. Plasmids were transformed in to competent *S. gordonii* as described

previously[149]. Expression of VHH on the surface of *S. gordonii* was verified using ELISA (A) and FACS (B).

A) ELISA: Based on the previously published protocol[150], wells of NUNC Maxisorp ELISA plates (Thermo Fisher Scientific Inc., Waltham, MA) were coated with 0.5 μg gD2 per well and VHH/Sg at various dilutions made in PBS were added ON at 4°C. After 4 x 200 μL washes with PBS-T, anti-myc (M. Matunis, Johns Hopkins, Baltimore, MD), anti-His (GE Healthcare Life Sciences, Piscataway, NJ), or anti-M (Siga Technologies, Corvallis, OR) primary antibody was added and incubated on the plate for 1 hr at RT. Cells were washed 4 x 200 μL with PBS-T and the appropriate secondary antibody (Jackson ImmunoResearch, West Grove, PA) was added. Cells were washed a final time with 4 x 200 μL PBS-T and binding was detected by adding 200 μL ABTS® ELISA HRP Substrate (KPL, Gaithersburg, MD). The plate was read at 405 nm using a BioTek Synergy HT Plate Reader (Winooski, VT).

B) FACS: A previously published flow cytometry protocol was used to further validate VHH surface expression on *S. gordonii*[151]. Samples were run on a Becton-Dickinson FACSCalibur Cytometer and data was analyzed using FloJo (Tree Star Inc., Ashland, OR).

HSV-2 Neutralization Assay with VHH and VHH/Sg

Vero cells were plated in Falcon 12-well trays (Thermo Fisher Scientific Inc., Waltham, MA) at $4x10^6$ cells per tray and incubated ON at 37°C. VHH samples (monovalent, bivalent, and pentavalent) were serially diluted in DMEM/2% FBS with HSV-2 G

(ATCC VR-734, Manassas, VA) at $5x10^3$ pfu/mL and all dilutions were incubated at 37°C for 1 hr. For VHH/Sg samples, bacteria were first fixed with 2% paraformaldehyde, washed, and resuspended at the appropriate concentration and virus was added at $5x10^3$ pfu/mL. Media was removed from the Vero cells and 100 µL of VHH dilutions with virus were added in duplicate to cells for 1 hr at 37°C, with gentle shaking every ten minutes to distribute liquid over cells. Cells were overlaid with 2 mL of 2% methylcellulose overlay/5% FBS in DMEM (Cellgro, Manassas, VA). Trays were incubated for 3 days at 37°C, stained with crystal violet, and plaques were counted.

Testing VHH and VHH/Sg Vaginal HSV-2 Animal Challenge Model

Six to eight week old female CF-1 were purchased from Harlan (Indianapolis, IN) and housed under reversed photoperiod conditions. As reported previously[152], mice are injected subcutaneously in the hindquarters with 2.5 mg of Depo Provera (UpJohn Co. 400 mg/mL) seven days before the planned viral challenge. On day seven, the VHH candidate (either purified VHH or VHH/Sg) and the viral inoculum of 10 ID_{50} are mixed in a total volume of 20 µL and promptly delivered to the vagina with a fire-polished Wiretrol pipet (Drummond Co., Broomall, PA). Three days later (Day 10) the vagina is lavaged using 20 µL of Bartel's Tissue Culture Refeeding Media; the fluid is delivered vaginally and withdrawn 10 to 20 times to collect HSV shed into the vagina. The lavage fluid is centrifuged at 6500 rpm for 5 minutes to remove mucus and cells, and then placed on human newborn foreskin cells to assay for presence of virus. Cells are observed by microscope 48 hours later and scored yes/no for infection. Mice used in these studies were maintained in accordance with the National Institutes of Health guidelines for the

humane use of laboratory animals. All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University (Protocol Number MO12H147).

Statistical Analysis

For the viral neutralization assays, the significance of the difference in plaque numbers was calculated using an ANOVA test, with a Bonferroni correction (STATA Corp, College Station, MD).

Results

Expression and Purification of Recombinant gD2 in Pichia pastoris

The sequence encoding the extracellular domain of gD2 was amplified from the HSV-2 strain 186 genome (Figure 2.2.A) and cloned in to the *Pichia pastoris* expression vector pPIC9 for expression and purification[153]. The size and purity of the purified gD2 was verified by separation with SDS-PAGE and staining with Coomassie (Figure 2.2.B), as well as by Western blot using a polyclonal anti-gD antibody (Figure 2.2.C). A band of approximately 48 kDa was detected with both methods, somewhat smeared due to the variable glycosylation pattern from *P. pastoris,* as has been reported previously[153]. gD2 was successfully detected by ELISA using a panel of conformational and non-conformational anti-gD2 antibodies (Figure 2.3), indicating that gD2 was successfully purified and suggests that it is folded correctly.

Monitoring Antibody Response of gD2 Immunized Llamas

Two llamas, Paquito and Rayo, were immunized five times with gD2, and after each immunization the animals were bled to obtain serum samples. The induction of anti-gD2 antibodies was determined using an ELISA. As shown in Figure 2.4, serum from both llamas demonstrated reactivity to gD2 following the second immunization compared to serum collected prior to immunization and after the first immunization. The highest reactivity to gD2 in serum collected from Rayo occurred after the fourth immunization. Serum from Paquito had similar reactivity to gD2 as Rayo, except there was a dramatic increase in gD2-reactivity following the last immunization (Figure 2.4). In addition to the ability to bind gD2, the serum was also tested for the ability to neutralize HSV-2. While

serum from Rayo had no significant neutralizing capability, Paquito's serum obtained after the fourth and fifth immunizations generated IC_{50} values of approximately 1:8 and 1:256, respectively (Figure 2.5). Taken together, these results indicate while both llamas did mount an antibody response against the gD2 immunogen as measured by ELISA, only Paquito developed a neutralizing antibody response. Since single domain antibodies are only a fraction of the total antibodies present in the llamas, this neutralizing response only guarantees that neutralizing antibody is present in the serum, and not that the neutralizing activity is due to single domain antibodies.

Construction of VHH/T7 Phage Display Library and Biopanning Against gD2

Based on the ELISA reactivity and neutralizing capability of the serum, VHH genes were amplified from cDNA generated from the PBMCs isolated after Rayo's fourth immunization and Paquito's fifth immunization (Figure 2.6). The amplified VHH genes were ligated into T7 phage vector to generate libraries with initial diversities of 3.9 x 10^7 pfu for Rayo and 1.98 x 10^7 pfu for Paquito. For Rayo, after the first round of biopanning, the titer of eluted phage increased stepwise following each round of biopanning, indicating a gradual enrichment for VHH-phage binding to gD2 (Figure 2.7). Titers of eluted phage from Paquito's library, however, reached saturation after only two rounds of biopanning (Figure 2.7), a pattern that was consistent despite repeated attempts with different conditions (data not shown). We interpreted this to mean that Paquito's library was already dominated by a population of phage reactive to gD2, and that further rounds of biopanning would not select for additional unique VHH sequences.

VHH-Phage Binding to gD2

Individual plaques from the phage elution after the second round of biopanning for Paquito and the sixth round of biopanning for Rayo were picked and amplified for analysis. Sixty VHH-phage clones were amplified from each llama and tested in an ELISA to determine if they can bind to gD2. For Rayo, of the 60 VHH-phage tested, 56 reacted to gD2 by ELISA (data not shown). After sequencing it was determined that 91% of these sequences were identical (R33), and that overall there were 6 unique VHH sequences (Figure 2.8). For Paquito, of the 60 VHH-phage tested, there were 48 VHH-phage that reacted to gD2 by ELISA (data not shown). Sequencing revealed that 94% of the VHH sequences were identical (P1), and that overall there were 4 unique VHH sequences (Figure 2.8). A VHH-phage clone, called P10, that was amplified from Paquito's library prior to any biopanning was also tested and sequenced for use as a negative control VHH-phage that did not bind to gD2. A standardized VHH-phage ELISA with 10^9 pfu per well was performed to determine relative reactivity to gD2 among the unique VHH isolated. All four of Paquito's unique VHH-phage had higher reactivity to gD2 than any of Rayo's VHH-phage, although all of them were higher than the negative control VHH-phage, P10 (Figure 2.9).

Antibody Capture Biopanning

Based on the antibody capture biopanning method described by Sanna et al[144], ELISA wells were coated with the non-neutralizing antibody DL6 in order to capture gD2 by binding to one of its non-neutralizing epitopes (Figure 2.10.A). After the first round of biopanning (Paquito's library only), the titer of eluted phage increased over the

next two rounds of biopanning (Figure 2.10.B), and plaques were picked after the third round to monitor the VHH sequences. 40 VHH-phage plaques were picked and tested for reactivity to gD2 by ELISA. Seventeen of these phage were able to bind gD2, and each of these phage were titered to perform a standardized phage ELISA with 10^9 pfu/well. As shown in Figure 2.10.C, their reactivity to gD2 was variable, but appeared to be higher than both R33/phage and P4/phage, which were run as positive controls. When the VHH genes were sequenced, however, all VHH sequences were identical to one of the 4 previously identified phage from Paquito.

VHH Sequence Analysis

An alignment of all 10 unique sequences that bind to gD2, plus P10, reveals that they are indeed VHH sequences and that the hallmark VHH residues are present (Figure 2.11). Comparing the sequences from each individual llama however, reveals limited variation in the sequences. For example, there are only 11 amino acid differences among the six unique VHH sequences for Rayo, and only five of those amino acid differences occur in variable regions of the VHH that determine antigen specificity, or complementarity determining region (called CDR). Many of the amino acid differences are conserved within the same class of amino acids and therefore unlikely to affect antigen binding. As a result, after determining that Rayo's VHH bind to gD2 at similar levels by ELISA (Figure 2.13), we decided to focus on R33, the majority sequence selected from biopanning.

Similarly, Paquito's VHHs exhibit only five amino acid differences, several of which are conserved within the same class of amino acids. Based on the VHH-phage

ELISA, we decided to proceed with P4, the VHH with the highest reactivity to gD2 in the VHH-phage ELISA (Figure 2.9). Paquito's sequences, while certainly VHH sequences, are missing several canonical amino acid residues (F37, E44, R45, G47), particularly in the very important framework 2 region (FR2). The FR2 region is critical for VHH folding and solubility because this is the region where the light chain would normally be interacting with the heavy chain, and is typically a very hydrophobic region in full-length antibodies. Camelid VHH antibodies, however, have evolved to accumulate amino acid changes that make the region more hydrophilic, allowing for the VHH to be soluble[154]. Interestingly, even though P10 was derived from Paquito, its framework sequences looks more like Rayo's, indicating that there were VHH sequences present in Paquito's original library with the correct framework regions, but biopanning did not favor selection of those sequences.

Purification of Monovalent and Bivalent VHH

VHH sequences were amplified from T7 phage as monovalent or bivalent (R33 only) VHH and cloned in to pET-47b for expression in *E. coli*. The purified VHH proteins were separated by SDS-PAGE for Coomassie staining to determine size and purity. All VHH derived from Rayo were located in the soluble fraction after induction, while all of Paquito's VHH were located in the pellet, as demonstrated in the representative gel in Figure 2.12.A. This observation is consistent with the sequence analysis that suggested the lack of conserved VHH residues in the FR2 region of Paquito's sequences might impact solubility. Proteins of approximately 15kDa for

monovalent VHH and approximately 30kDa for bivalent VHH were detected (Figure 2.12.B).

Binding of Monovalent and Bivalent VHH to gD2

The ability of the purified VHH to bind to gD2 was tested by ELISA (Figure 2.13). Surprisingly, despite the high binding of Paquito's VHH to gD2 when expressed as a fusion protein on the surface of the phage, purified VHH from Paquito show minimal binding to gD2. Conversely, while VHH-phage from Rayo bound poorly to gD2, when expressed and purified, R33 exhibits superior binding ability. There does not appear to be enhanced binding with bivalent R33 compared to monovalent R33 when added in equimolar amounts. It is possible that expression of Paquito's VHH as a fusion protein with phage proteins forces the VHH to fold in a way that promotes binding, but when these same VHH sequences are expressed by *E. coli* as a monomeric proteins, they are no longer constrained by flanking phage protein and are unable to fold and bind to gD2.

The same pattern of gD2 binding was also demonstrated by flow cytometry. We used flow cytometry analysis with z4/6[148] cells that express gD2 on their surface as a way to measure native gD2 expressed at the cell surface. All VHH derived from Rayo, including the bivalent R33, were again able to bind z4/6 cells, while P4 was unable to bind (Figure 2.14).

Pentavalent VHH

Based on increasing evidence that VHH have enhanced activity when expressed in multivalent context[155], we expressed R33 as a fusion protein with the verotoxin B

subunit, which allows for self-assembly into a pentamer[147]. The verotoxin B subunit was fused to the N-terminus of R33 (NR33) and purified (Figure 2.17.A). To verify that the NR33 did self-assemble, a sample of the purified NR33 was run through a Superdex200 column and it was found that they eluted at a peak of about 100kDa, roughly the size of the expected protein (data not shown). When corrected for valency, NR33 was able to bind to gD2 as measured by ELISA at similar levels compared to monovalent R33 (Figure 2.13.B).

Surface Expression of VHH on S. gordonii (VHH/Sg)

R33 and P10 were cloned in to the pLEX plasmid for expression as a fusion protein with a surface protein of *S. gordonii*, called the M-protein[149]. Transformants were tested for VHH expression with flow cytometry by detection with anti-myc antibody. Compared to vector transformed (pLEX) *S. gordonii*, both R33 and P10 expression can be detected on the surface of *S. gordonii* (Figure 2.15). Attempts at identifying a positive P4 transformant were unsuccessful, possibly due to the insoluble nature of Paquito's sequences. An ELISA was performed to determine if the surface expressed R33 was functional and could still bind to gD2. Binding of VHH/Sg was detected using an anti-myc antibody. As shown in Figure 2.16, *S. gordonii* expressing R33 bound to gD2, while P10/Sg and vector transformed *S. gordonii* did not exhibit any gD2 binding.

VHH and VHH/Sg Neutralization Assay

All forms of the VHH (monovalent, bivalent, pentavalent, and expressed on the surface of *S. gordonii*) were tested in an HSV-2 neutralization assay. R33, bvR33, P4

were unable to neutralize the virus at the concentrations tested (Figure 2.18.A and B). Additionally, R33/Sg did not neutralize the virus compared to P10/Sg or vector transformed *S. gordonii* (pLEX/Sg) (Figure 2.20). Neutralization assays with pentameric R33 showed that NR33 does have neutralizing activity, and that the inhibition compared to untreated virus is statistically significant at several of the dilutions tested (Figure 2.18.C). At the highest amount tested (10uM), NR33 neutralized the virus at 57% and the inhibition remained statistically significant until 0.1uM. Protein precipitation at higher concentrations prevented testing neutralization activity at higher levels of pentavalent VHH from being tested, so it is not possible to calculate an IC_{50} for NR33 from the completed experiments, but it is clear from multiple experiments that the inhibition is consistent and statistically significant. Further protein purification troubleshooting would likely allow for higher concentrations to be evaluated.

Testing VHH and VHH/Sg in Vaginal HSV-2 Animal Challenge Model

At the time of animal testing, the gD2-binding VHH candidates that were available were R33, bvR33, and R33/Sg. P10 and P10/Sg were used as the negative controls. Equivalent amounts of R33 and P10 or equivalent concentrations R33/Sg and P10/Sg were mixed with virus and introduced in to the mouse vagina to determine if the VHH had any HSV-2 *in vivo* neutralizing capability. As showed in Table 2.1, none of the VHH were able to prevent infection.

Discussion

The overarching goal of this thesis work was to isolate an anti-gD2 VHH that could neutralize HSV-2 and act as a microbicide to protect against vaginal transmission of HSV-2 with delivery by the model commensal bacteria *Streptococcus gordonii*. gD2 was chosen as the target for the virus based on extensive research indicating that neutralizing antibodies directed against this protein could prevent HSV-2 infection *in vitro* and *in vivo*, including a study demonstrating that an anti-gD2 scFv could prevent HSV-2 infection in a genital challenge guinea pig model[6]. This is significant because scFvs, like VHHs, lack the Fc region of full-length antibodies (see Figure 2.1), indicating that the antigen binding capacity of the scFv was sufficient to block the virus. Large-scale expression and purification of properly folded scFvs is often problematic, however. The small size, ease of purification, and stability under a wide range of conditions make VHH ideal candidates for an HSV-2 microbicide directed against a neutralizing epitope of gD2.

After immunizing two llamas with gD2 and verifying that the llamas mounted immune responses against the antigen, two phage libraries were constructed that displayed the VHH repertoire of each immunized llama. The libraries were biopanned against gD2, and 10 unique VHH were identified and investigated. Initial ELISA results indicated that the four VHH-phage isolated from Paquito were superior at binding to gD2 compared to the six VHH-phage derived from Rayo. Upon expression and purification in *E. coli*, however, it became apparent that Paquito's VHH bound very weakly to gD2, while Rayo's VHHs bound very strongly. Further analysis of VHH sequences derived from Paquito revealed that several key residues conserved across all VHH genes in the

FR2 region were not present, perhaps leading to insoluble VHH that could not fold correctly.

Initial *in vitro* evaluation of the six VHH from Rayo indicated that they were functionally equivalent. Based on the lack of significant sequence diversity and the similar gD2-binding capability as demonstrated by ELISA and FACS, the sequence that was present at the highest frequency after biopanning, R33, was chosen as the main VHH to continue testing. Although R33 was the most extensively tested for neutralizing capability, all of the other unique VHH (including Paquito's) were tested at least once in the *in vitro* HSV-2 neutralization assay (data not shown), and none of them demonstrated any neutralizing activity (Figure 2.18.A). Additionally, R33 was tested in a mouse model used to evaluate HSV-2 microbicide efficacy, and it did not offer any protection against HSV-2 infection (Table 2.1). There is still a great deal of interesting characterization that could be done with R33, particularly determining which epitope of gD2 that it binds to. Since the outcome we were investigating was HSV-2 neutralization, however, we did not undertake these studies.

Due to the known neutralizing capability of antibody present in Paquito's serum following immunization (Figure 2.5), an alternative biopanning strategy was used to try to isolate additional unique VHH sequences from Paquito's library. Using a protocol described by Sanna et al[144], we performed antibody capture biopanning with the non-neutralizing antibody DL6 to promote the selection of VHH-phage that bind to a gD2 neutralizing epitope. This method of biopanning only selected for VHH that we had previously identified, which only definitively proves that DL6 does not bind to the same epitope that the initially selected VHH do. Furthermore, this result suggests that the

epitope recognized by the four VHH from Paquito that were initially identified through the regular biopanning strategy is the immunodominant epitope to which Paquito's immune system responded and that the functional diversity of VHH sequences in Paquito's library is low. Time constraints forced us to continue with evaluating the VHH candidates that we had identified rather than repeatedly biopanning to obtain new VHHs. However, a possible next step would be to perform biopanning with gD2 peptides that correspond to known neutralizing epitopes to eliminate the possibility of selecting for VHH-phage that bind to non-neutralizing epitopes.

When it became evident that monomeric VHH were not able to neutralize the virus, we explored ways to create multimeric VHH to see if that could impart neutralizing activity. Reports indicating that monomeric VHH may not be as potent as once thought prompted us to first construct bivalent R33, to mimic the bivalent nature of regular antibodies. Bivalent R33 bound to gD2 at similar levels as monomeric R33 (Figures 2.13 and 2.14) and likewise did not demonstrate neutralization activity (Figure 2.18.B). Expression of VHH on the surface of *S. gordonii* had been shown to make VHH effectively multivalent and increase the efficacy of VHH[141]. Expression of R33, however, on the surface of *S. gordonii* did not confer neutralizing capability *in vitro* (Figure 2.19.B) or *in vivo* (Table 2.1). Although R33 was readily detectable on the surface of *S. gordonii*, it is possible that R33 was not expressed at a high enough density on the surface of the bacteria to achieve a multivalent effect.

As a last effort to explore the potential antiviral activity of R33, we expressed R33 as N-terminal fusion with verotoxin B subunit to allow for pentamerization of VHH. Previous research had demonstrated that a pentameric VHH have increased avidity for its

antigen compared to monomeric VHH, are very thermostable, and also were resistant to cleavage by many proteases[147]. The pentameric version of R33 resulted in neutralizing activity that was significantly greater than R33 or virus alone at the three highest dilutions tested. While the IC_{50} of NR33 could not be calculated due to protein concentration limitations, NR33 consistently neutralized 50% of the virus at approximately 1uM. While pentameric VHH are structurally not as simple as monomeric VHH, it is possible that they could still be a viable microbicide candidate because their complexity results from a self-assembly reaction and is not dependent on correct disulfide bond formation or folding during protein purification. While it may be difficult to achieve high enough concentrations for efficient self-assembly if secreted extracellularly by a commensal bacteria, very high levels of the pentameric VHH could likely be produced *in vitro* for vaginal application by another delivery mechanisms.

Despite minimal neutralizing activity, there remain potential applications for which monomeric R33 can be used, two of which are explored in the next chapters of this thesis. Due to its small size, R33 could be used in microscopy studies to visualize gD2 in the infected cell. Additionally, R33 could be used clinically as a diagnostic reagent. Since R33 can be easily generated at mg/mL concentrations in *E. coli* and has poor reactivity to HSV-1 (data not shown), it is theoretically possible to adapt R33 as a diagnostic test to differentiate between HSV-1 and 2 infections. VHH are potentially extremely valuable biomedical tools, and using a VHH to target a neutralizing epitope of gD2 was certainly a feasible goal. Through biopanning we were able to identify 10 unique VHH that bind to gD2, but we unfortunately were unable to pull the neutralizing VHH “pin” out of the phage display library “haystack.”

Figure 2.1: Antibody Structure
Structural comparison of human and camelid antibodies and antibody fragments. Adapted from Holliger & Hudson 2005.

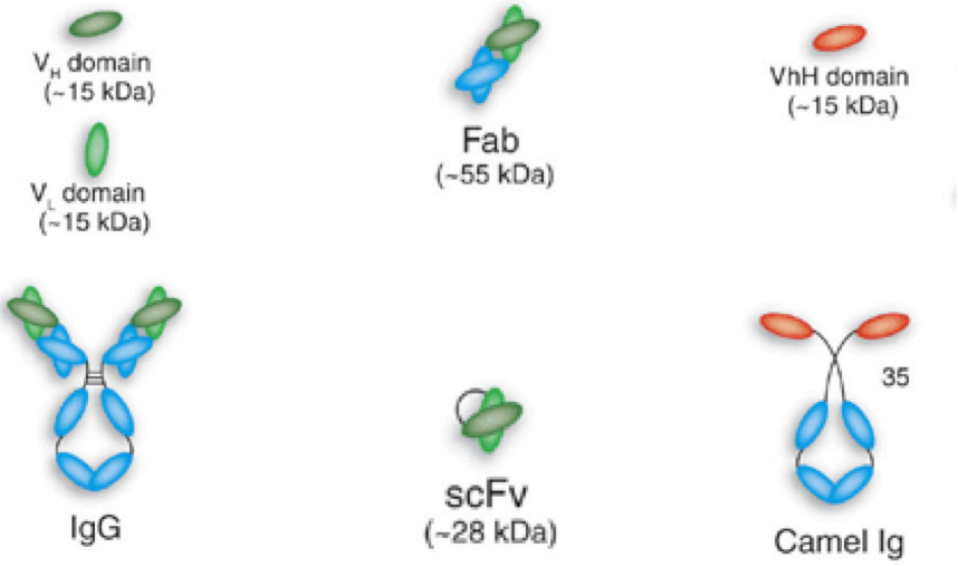


Figure 2.2: Purification of gD2 From *Pichia pastoris*

A) DNA encoding amino acids 1-314 of gD2 were amplified by PCR from the HSV-2 strain 186 genome. B and C) Purified gD2 from Pichia pastoris was separated by SDS-PAGE and stained with Coomassie Brilliant Blue (B) or transferred to PVDF membrane and detected with a polyclonal anti-gD2 antibody (R45) by western blotting (C).

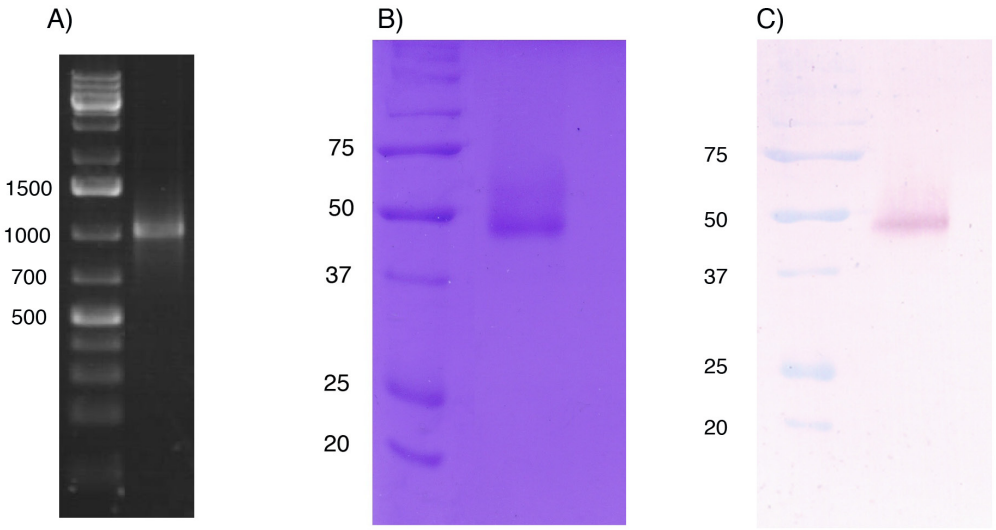


Figure 2.3: Antibody Reactivity to Purified gD2 (ELISA)

ELISA wells were coated with gD2 and detected with a panel of anti-gD2 antibodies: R45 (rabbit, polyclonal), HSV8 (human, monoclonal), DL6 (mouse, monoclonal), anti-His (mouse, monoclonal). Additionally wells coated with gD2 where only HRP-conjugated secondary antibody (anti-rabbit, anti-human, and anti-mouse) was added were run as controls.

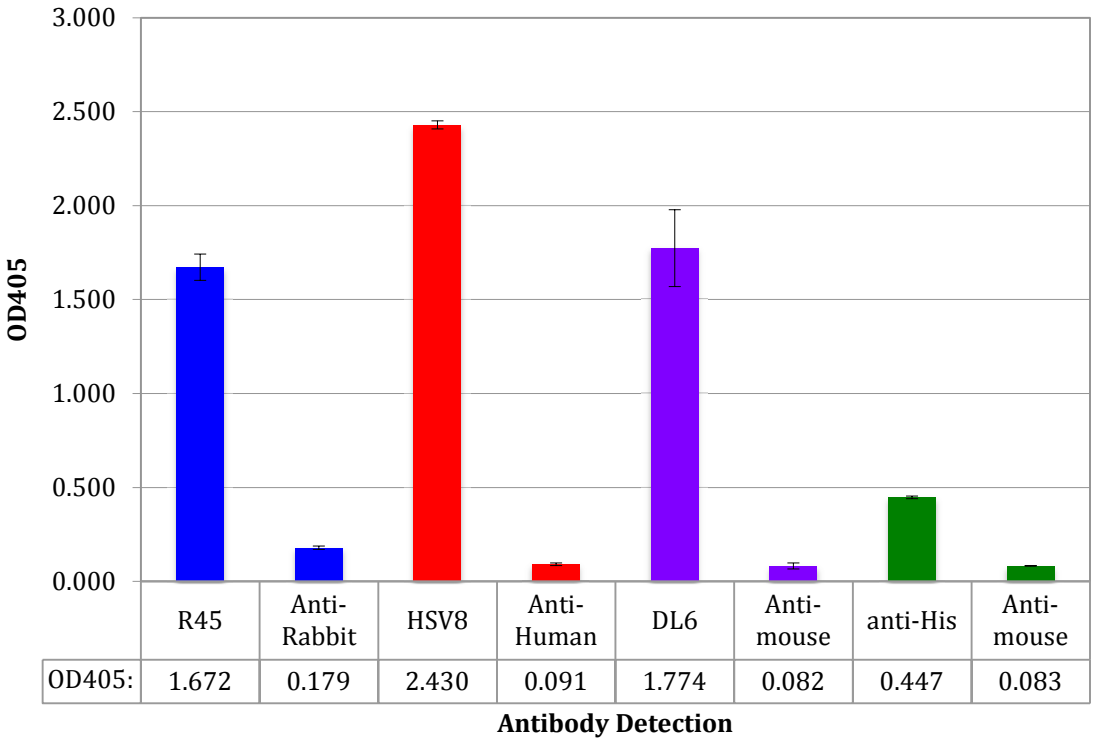


Figure 2.4: Llama Serum ELISA

Llama serum collected before the initiation of immunization (naïve) and after each immunization (Im# 1-5) was diluted 1:10,000 and used to coat ELISA wells. gD2 was added and binding was detected to determine if the llamas mounted an immune response against the gD2 immunizations.

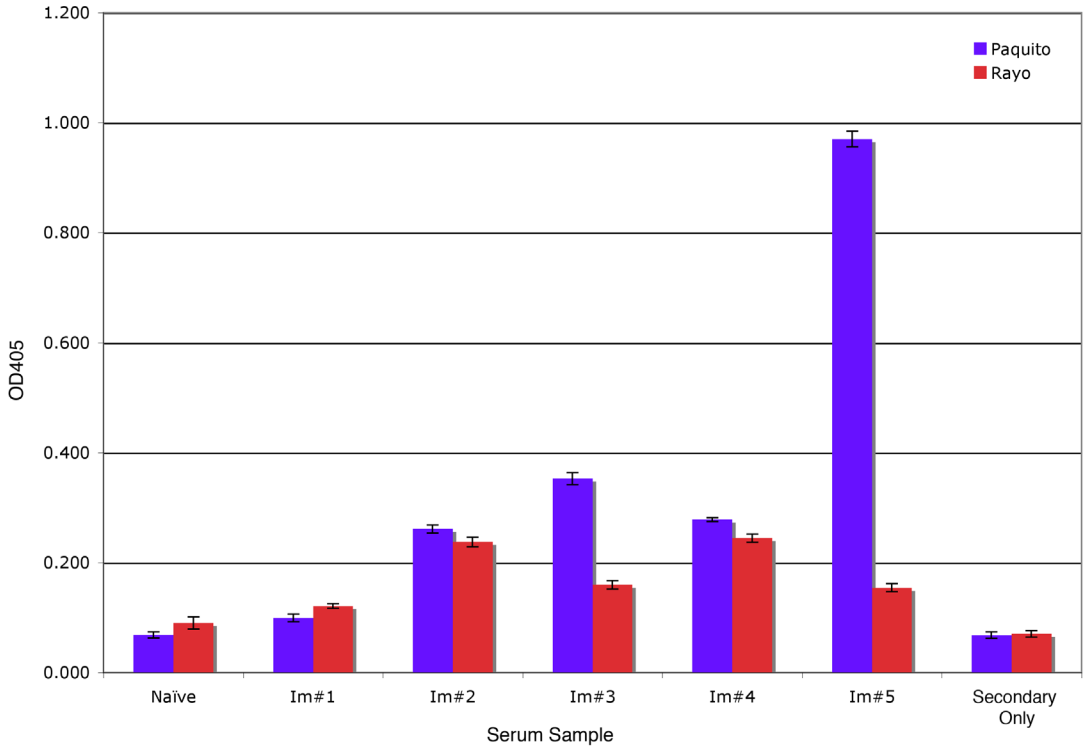


Figure 2.5: Llama Serum Neutralization Assay

Naïve llama serum and llama serum following the 4th and 5th immunizations was serially diluted and incubated with HSV-2 for 1 hr at 37°C before adsorption on Vero cells for 1 hr. After overlay with methylcellulose and incubation for 2 days, cells were stained and plaques counted. Each dilution was assayed in duplicate and error bars represent maximum and minimum percent values. Results were expressed as percent neutralization compared to naïve serum.

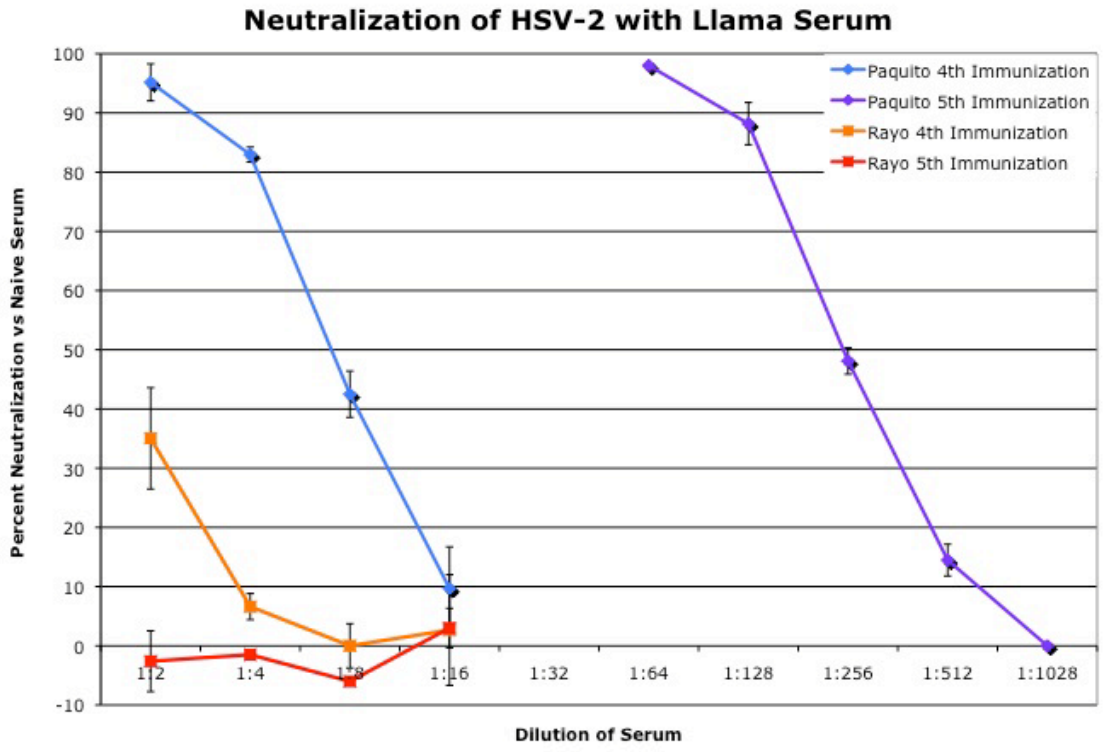


Figure 2.6: Amplification of VHH Regions from Llama DNA

After the final immunization, PBMCs were separated from whole blood and RNA was purified for synthesis of cDNA. Nested primer sets[143] were used to amplify the cDNA sequence of the variable region from heavy chain only antibodies (VHH), and PCR products were separated by agarose gel electrophoresis. Labels indicate which llama and round of PCR the sample is derived from.

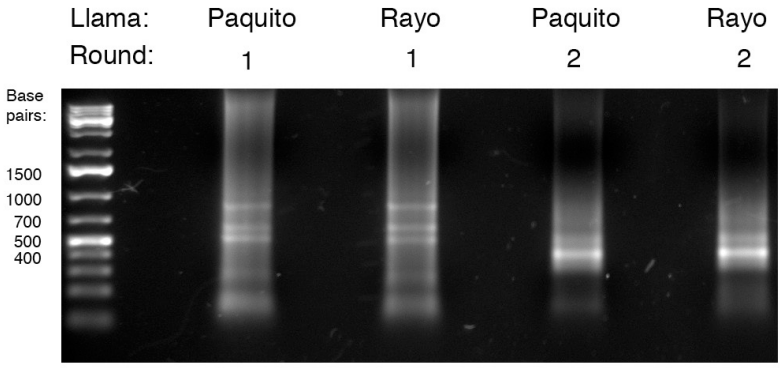


Figure 2.7: Biopanning of VHH-Phage Library on gD2

After each round of biopanning, the eluted phage were titered to monitor the concentration of phage during the biopanning process. The large drop in phage titer after the first round of biopanning was expected, as most of the VHH-phage in the library are not specific for gD2. Subsequent rounds of biopanning show increased phage concentration as enrichment for gD2 specific VHH-phage occurs.

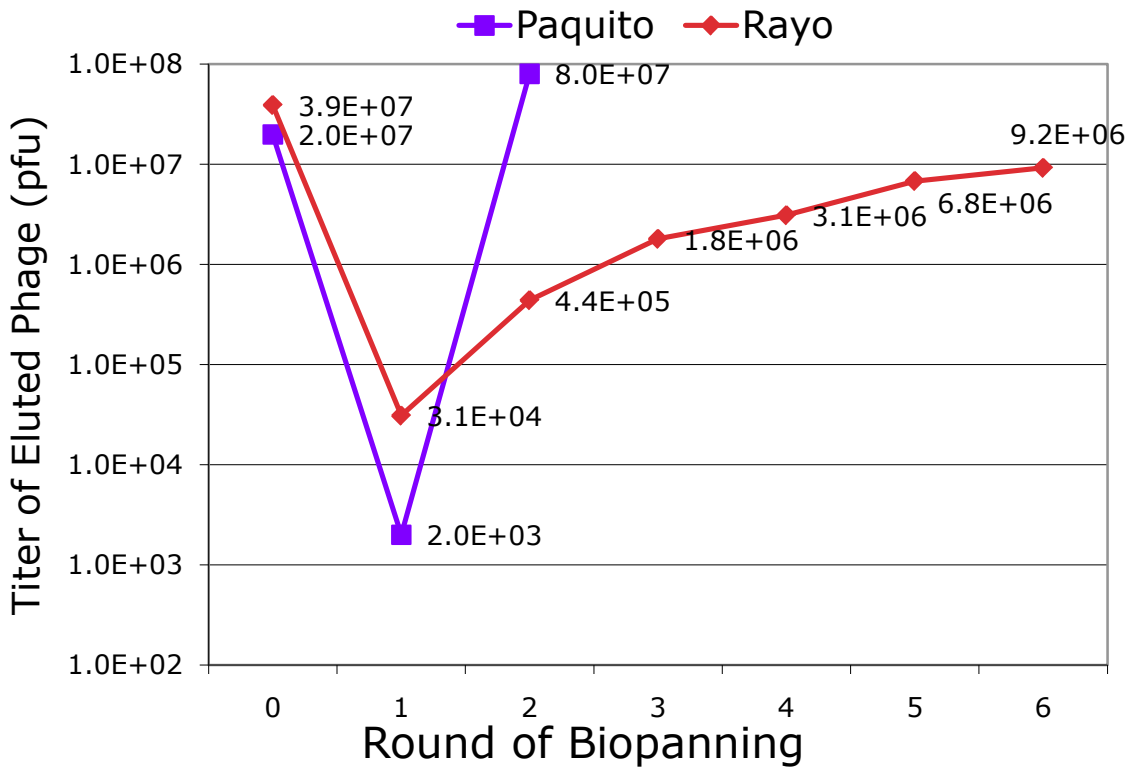


Figure 2.8: VHH Sequence Flowchart

Flowchart diagramming the process of identifying unique VHH sequences that bind to gD2. Individual phage clones were amplified and tested by ELISA to determine if they are reactive to gD2. Those that were reactive were sequenced, and the sequences were compared to determine the number of unique VHH sequences.

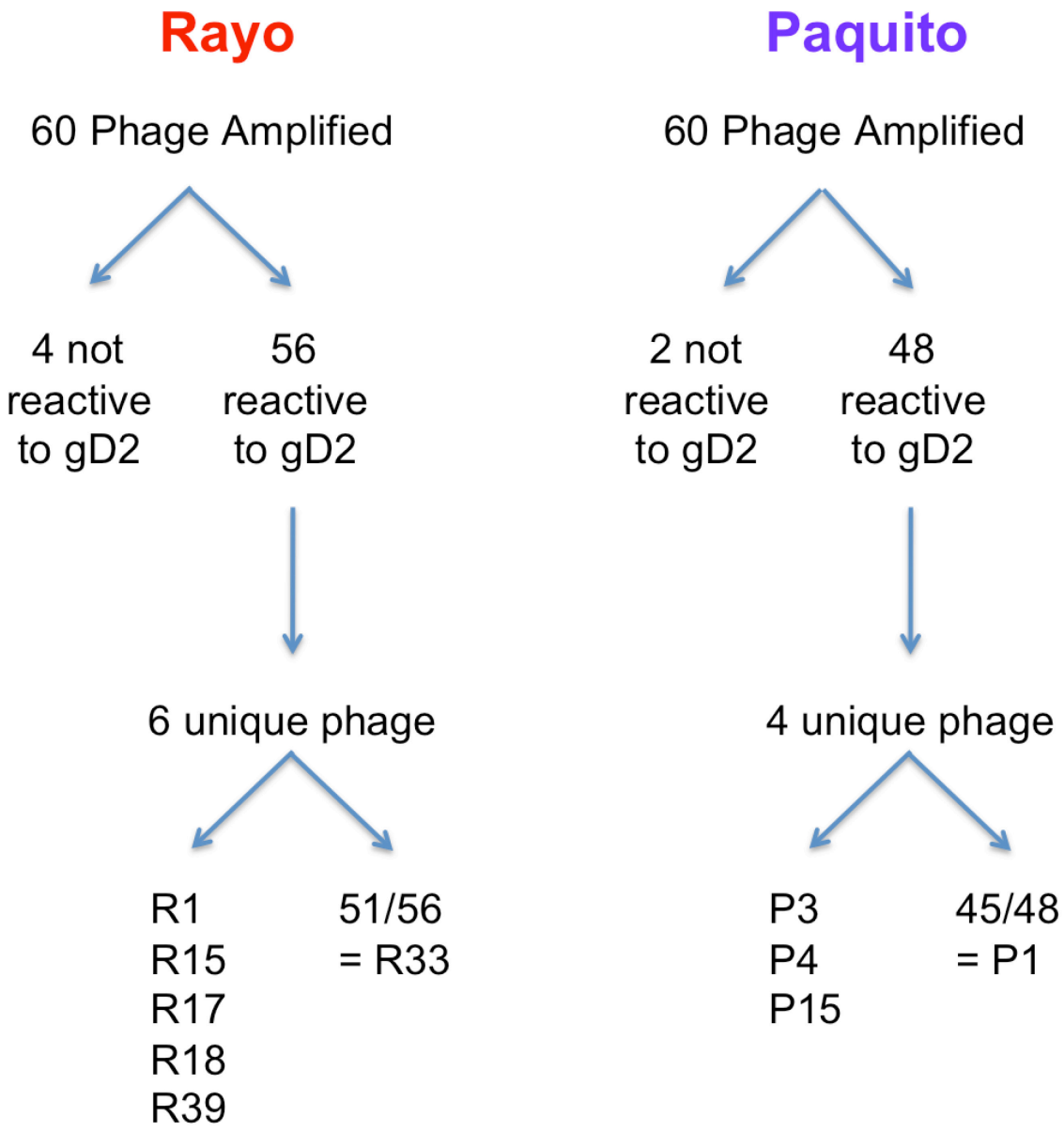


Figure 2.9: VHH-Phage Binding to gD2

VHH-phage clones after multiple rounds of biopanning were individually amplified and tested for reactivity to gD2 by ELISA. Wells were coated with gD2, VHH-phage clones were added and then detected with an anti-phage antibody. The six unique VHH from Rayo (R1, R15, R17, R18, R33, and R39), four unique VHH from Paquito (P1, P3, P4, and P15), and one non-gD2 binding VHH-phage (P10) were tested.

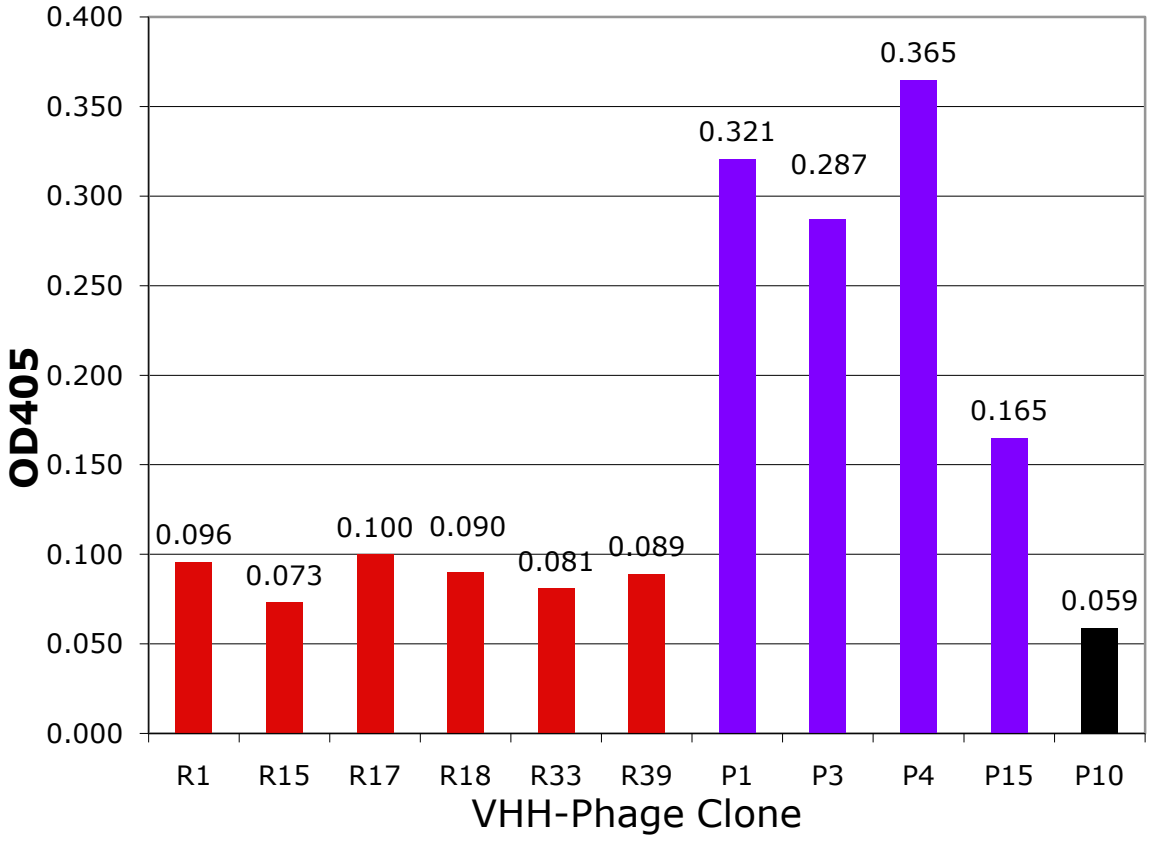
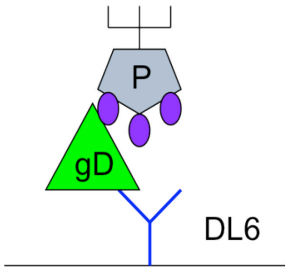


Figure 2.10: Antibody Capture Biopanning

A) Conceptual diagram of how capture biopanning immobilizes gD2 through binding to a particular epitope, thereby promoting selection of sVHH-phage that bind to other sites of gD2. B) The eluted phage after each round of biopanning were titered to monitor the concentration of phage during the biopanning process.

A)



B)

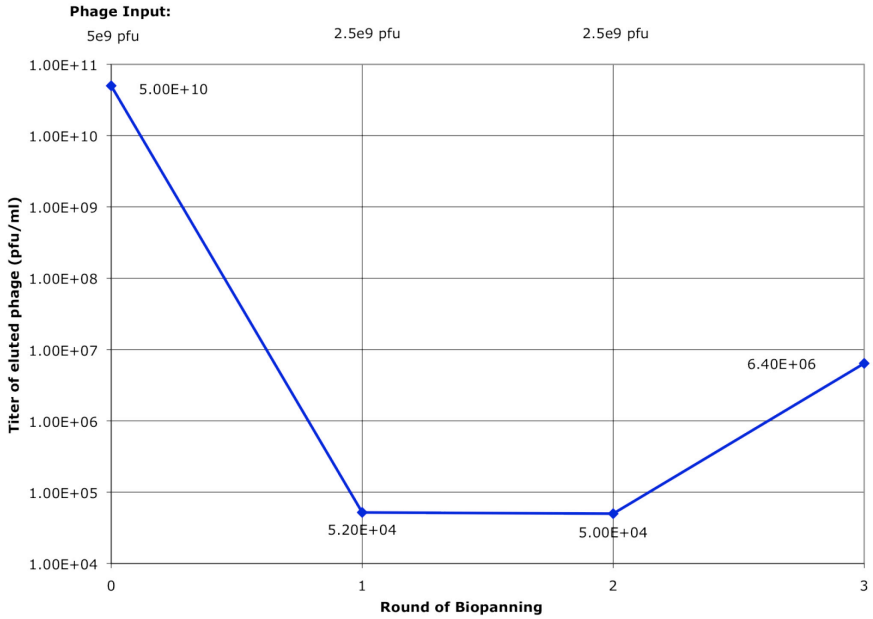


Figure 2.10: Antibody Capture Biopanning VHH-Phage ELISA

VHH-phage clones after three rounds of capture biopanning with Paquito's phage display library were individually amplified and tested for reactivity to gD2 by ELISA. Wells were coated with gD2 and VHH-phage clones were added and then detected with an anti-phage antibody. Previously identified VHH-phage were used as positive (R33 and P4) and negative (P10) controls. The anti-gD2 antibody DL6 was also used as a positive control. Each VHH-phage was assayed in duplicate and error bars represent maximum and minimum values.

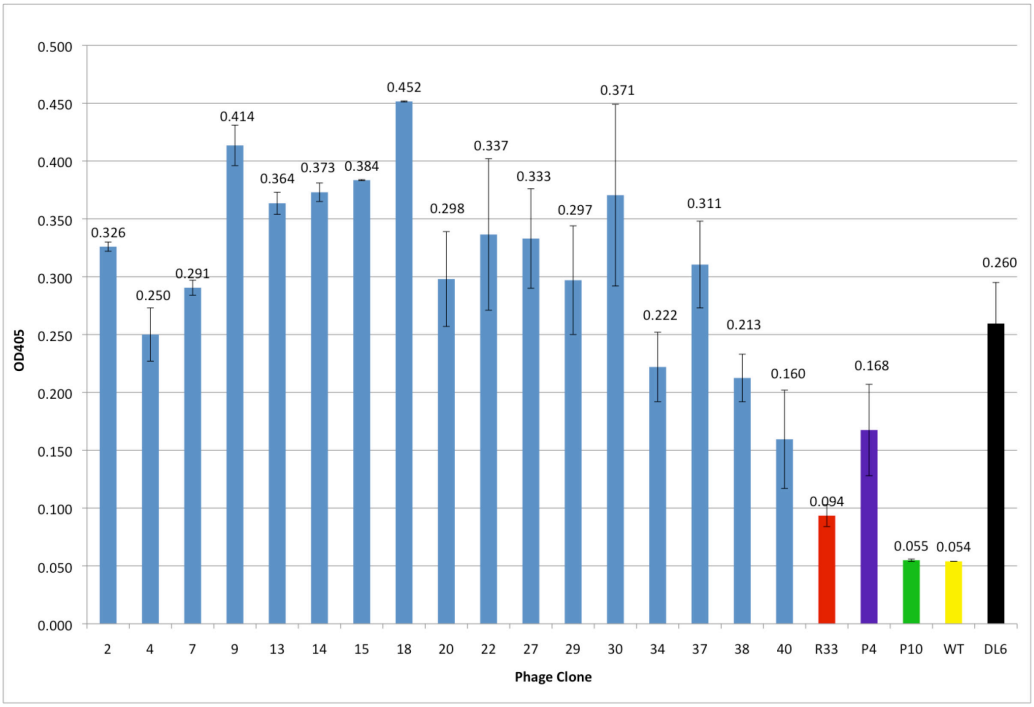


Figure 2.11: Unique VHH Amino Acid Sequence Alignment

VHH inserts, originally amplified from variable region of heavy chain only antibodies, were sequenced from VHH-phage clones and aligned to determine unique VHH sequences identified from the gD2 biopanning process.

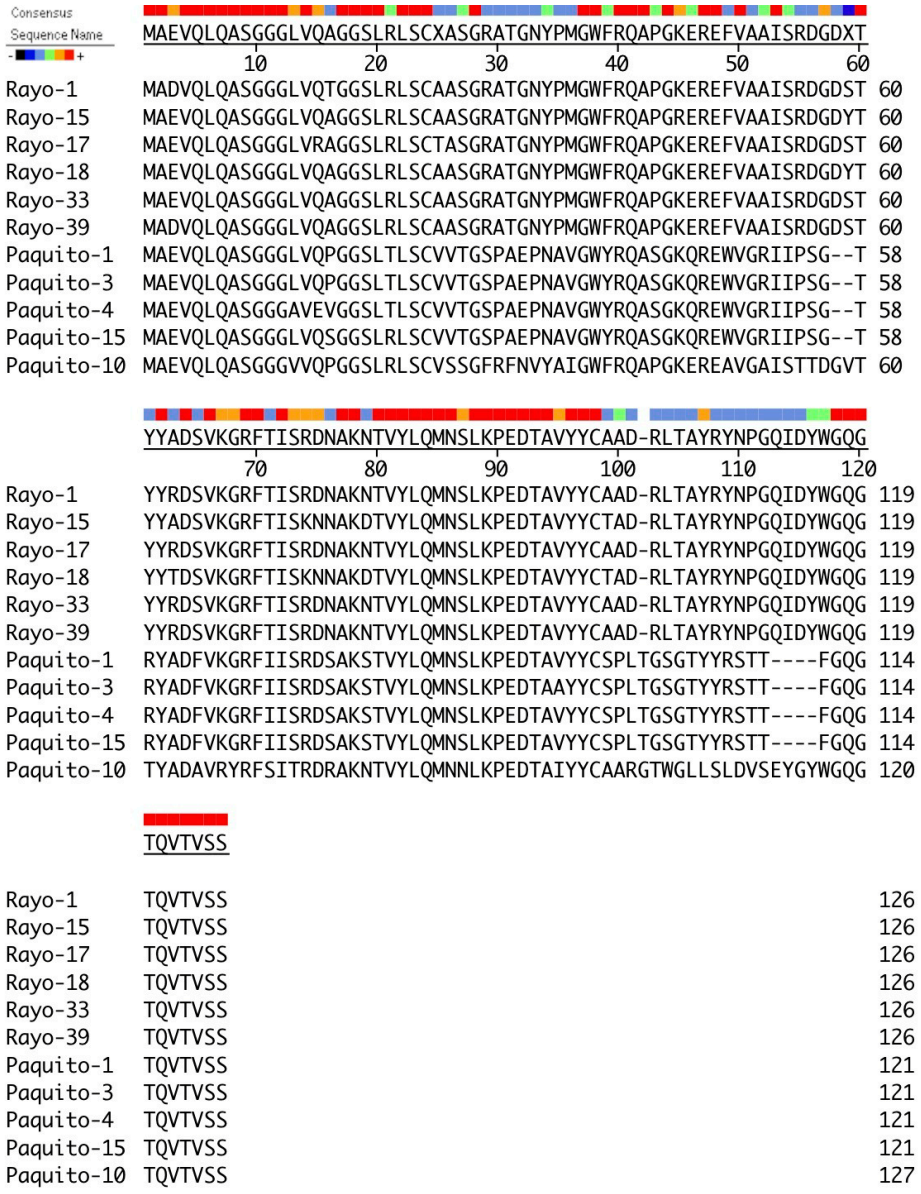
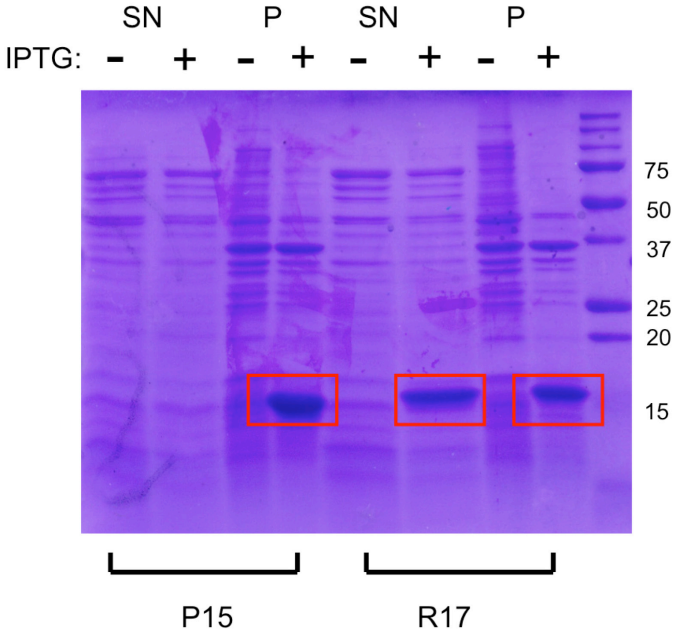


Figure 2.12: Expression and Purification of VHH from *E. coli*

A) *E. coli* were transformed with VHH/pET plasmids and small scale cultures were grown and induced to determine solubility of VHH proteins. A representative gel demonstrating that VHH derived from Paquito are located in the pellet (P), while VHH derived from Rayo are located in both the supernatant (SN) and the pellet. B) A representative gel demonstrated the size and purity of purified R33 and bvR33.

A) Evaluating VHH Solubility



B) Purified bvR33 and R33

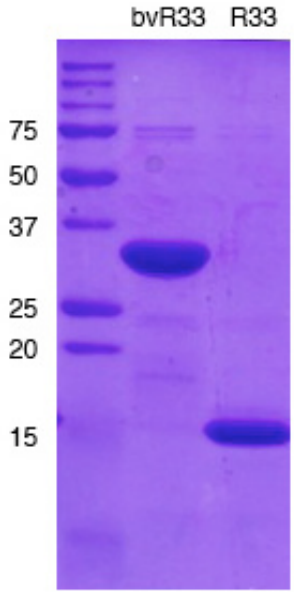
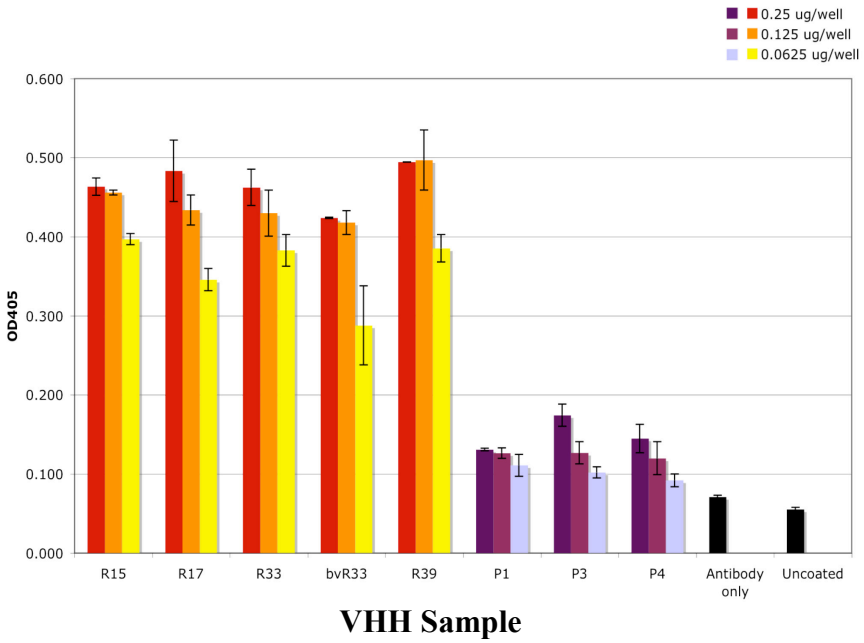


Figure 2.13: Purified VHH Bind to gD2

ELISAs A) and B) were performed in which wells were coated with the indicated VHH and gD2 was added to assay for their ability to bind gD2. Each dilution was assayed in duplicate and error bars represent maximum and minimum values.

A)



B)

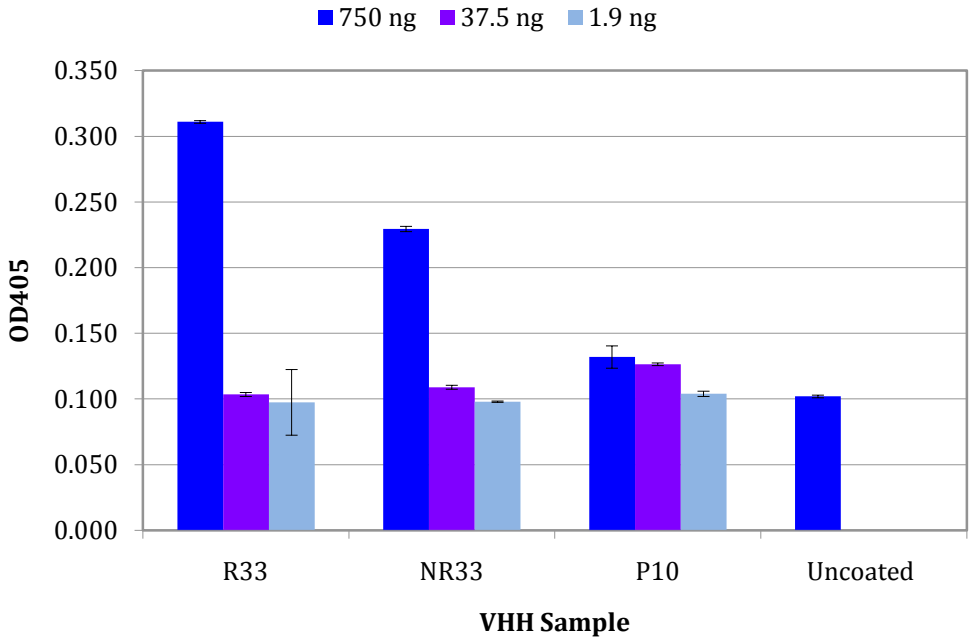


Figure 2.14: VHH Binding to gD2-Expressing Cell Line

To determine if VHH could bind to gD2 expressed at the cell surface, z4/6 cells (surface expression of gD2) were stained with various VHH (C: R33, D: P4, E: bvR33, F: R15) and detected by a FITC-conjugated secondary antibody. DL6 was used as a positive control to verify that gD2 was expressed (A), and a secondary antibody control with no VHH or primary antibody was also used as a negative control (B).

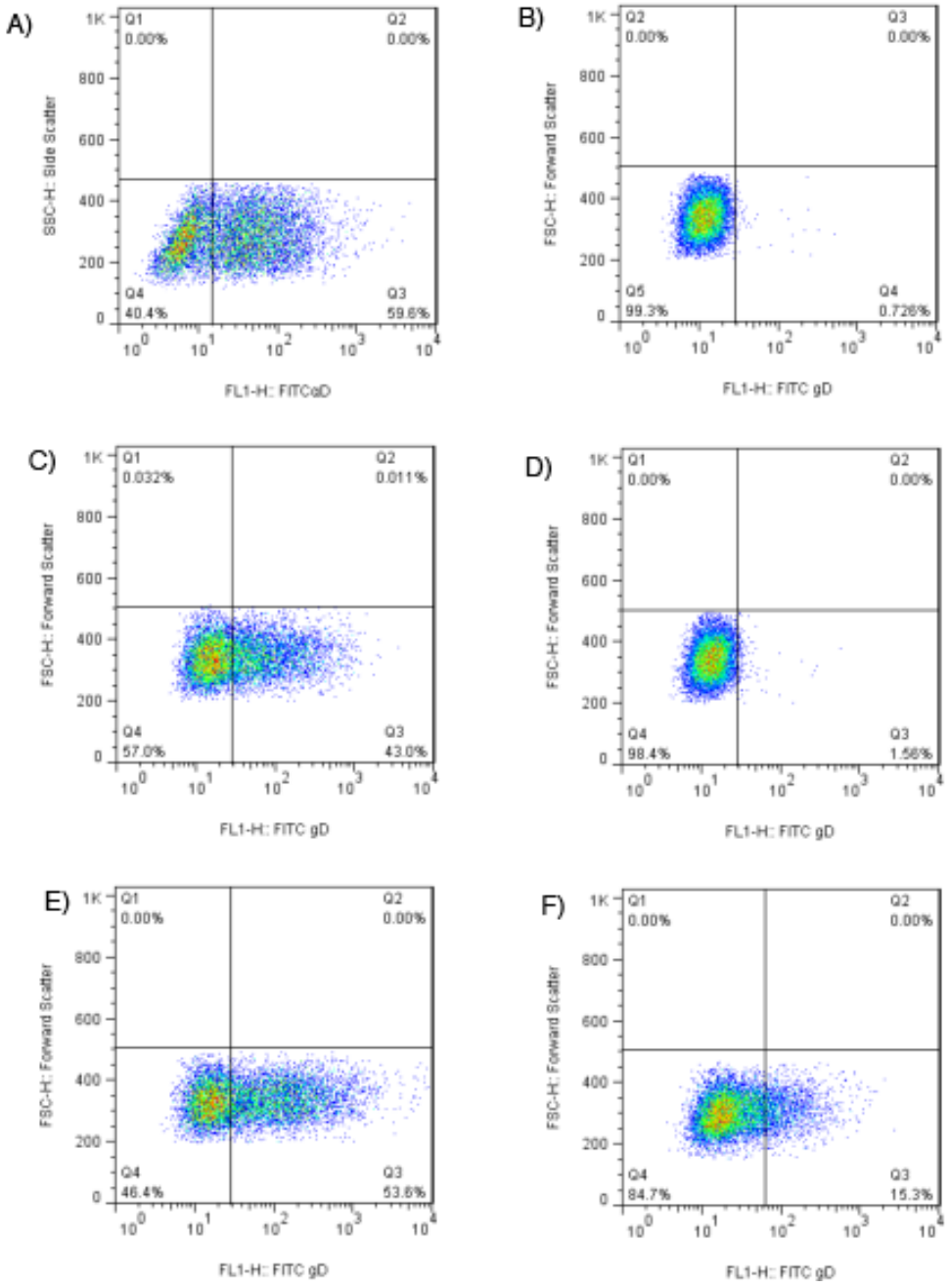


Figure 2.15: FACS to Validate VHH Expression on *S. gordonii*

S. gordonii transformed with the pLEX vector (red), P10/pLEX (orange), and R33/pLEX (blue) were stained with anti-myc to detect VHH expression at the surface of the bacteria using standard flow cytometry protocols.

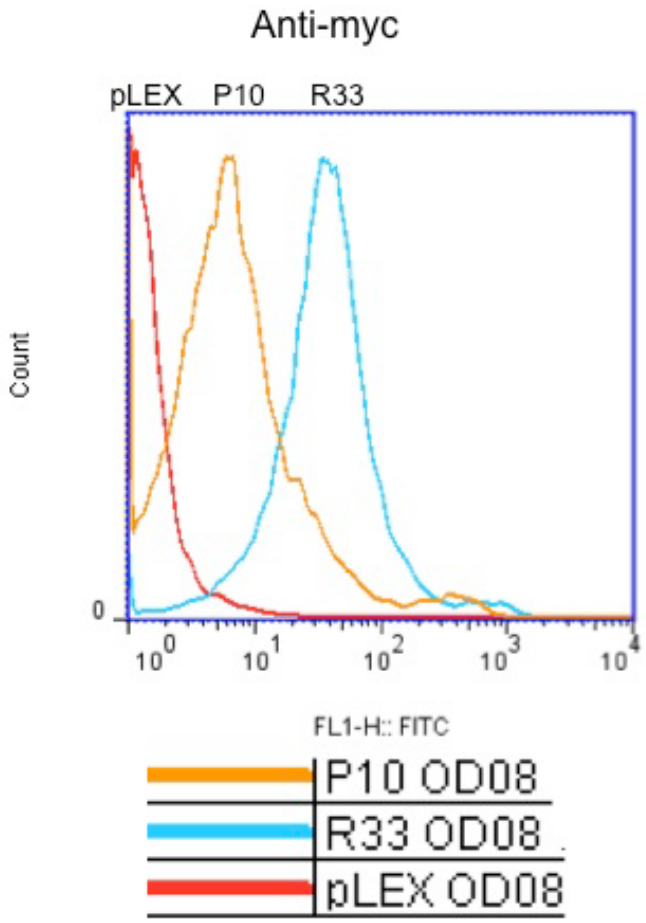


Figure 2.16: ELISA to Validate Functional VHH Expression on *S. gordonii*

An ELISA was performed in which wells were coated with gD2 and then either transformed S. *gordonii* expressing R33 or P10 or vector transformed *S. gordonii* (negative control) were added to determine if the presence of VHH at the bacterial surface allowed for binding to gD2. Each dilution was assayed in duplicate and error bars represent maximum and minimum values.

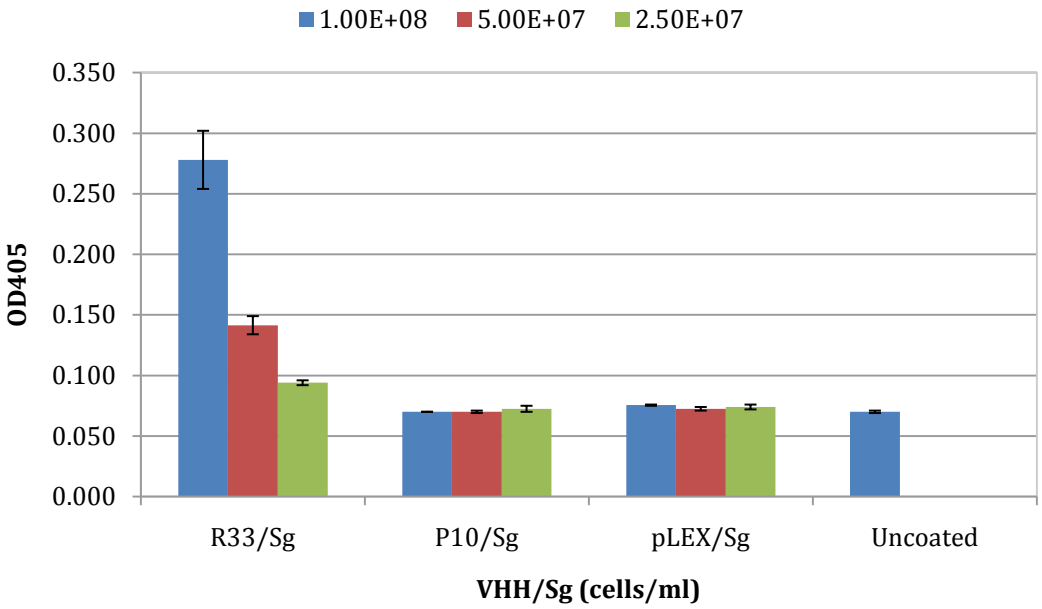


Figure 2.17: Purification of Pentavalent VHH

R33 expressed as a fusion protein with the verotoxin B subunit (NR33), allowing for pentamerization, were purified from transformed *E. coli* and separated by SDS-PAGE for staining with Coomassie to determine size and purity. Upon dialysis, the monomers self-assemble in to a pentamer.

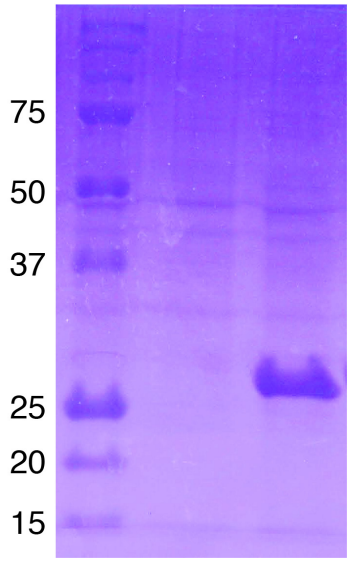
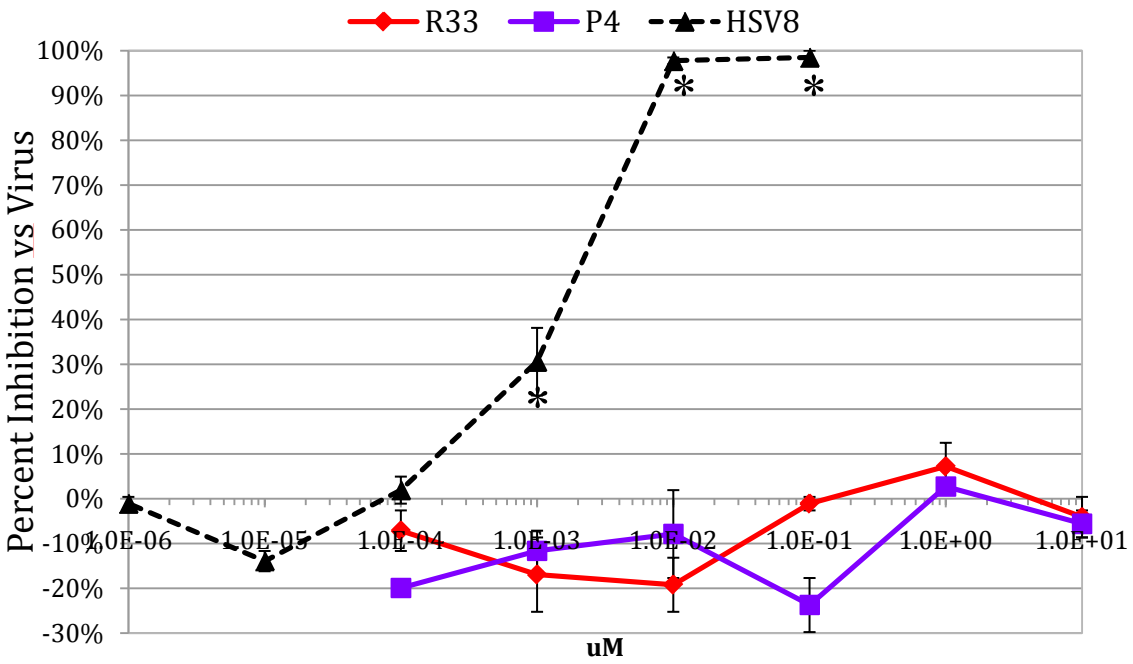


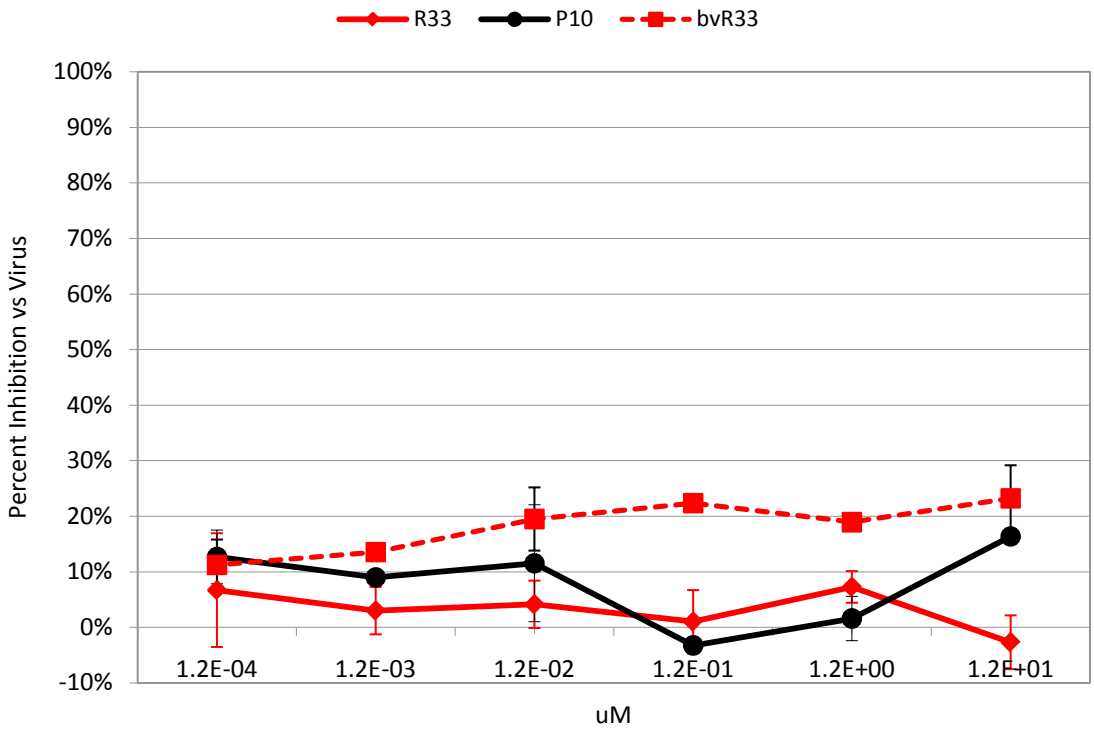
Figure 2.18: VHH Neutralization of HSV-2

Virus was incubated with dilutions of VHH for 1 hr at 37°C and then plated on Vero cells to assay for VHH neutralizing activity. Each dilution was assayed in duplicate and error bars represent maximum and minimum plaque numbers. Results are expressed as percent inhibition compared to plaque numbers from untreated virus. Statistical significance compared to untreated virus was calculated by ANOVA and is indicated by asterisks ($P < 0.05$). The known neutralizing antibody HSV8 was used in graph A as a positive control.

A) Monovalent VHH Neutralization Assay



B) Bivalent R33 Neutralization Assay



C) Pentavalent VHH Neutralization Assay

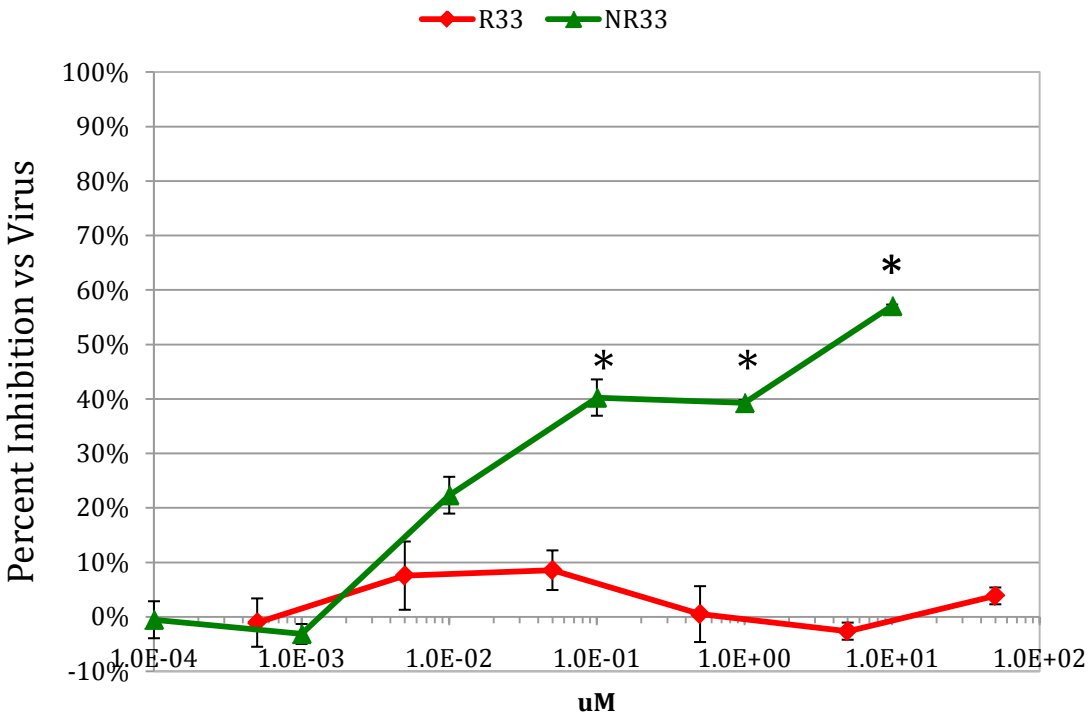


Figure 2.19: VHH/*S. gordonii* Neutralization Assay

Dilutions of transformed S. *gordonii* expressing R33 or P10 or vector transformed *S. gordonii* were fixed with paraformaldehyde, washed, and incubated with virus for 1 hr at 37°C. Each dilution was assayed in duplicate and error bars represent maximum and minimum plaque numbers. Results are expressed as percent inhibition compared to plaque numbers from untreated virus. None of the dilutions were statistically significant than virus treated with pLEX transformed *S. gordonii*.

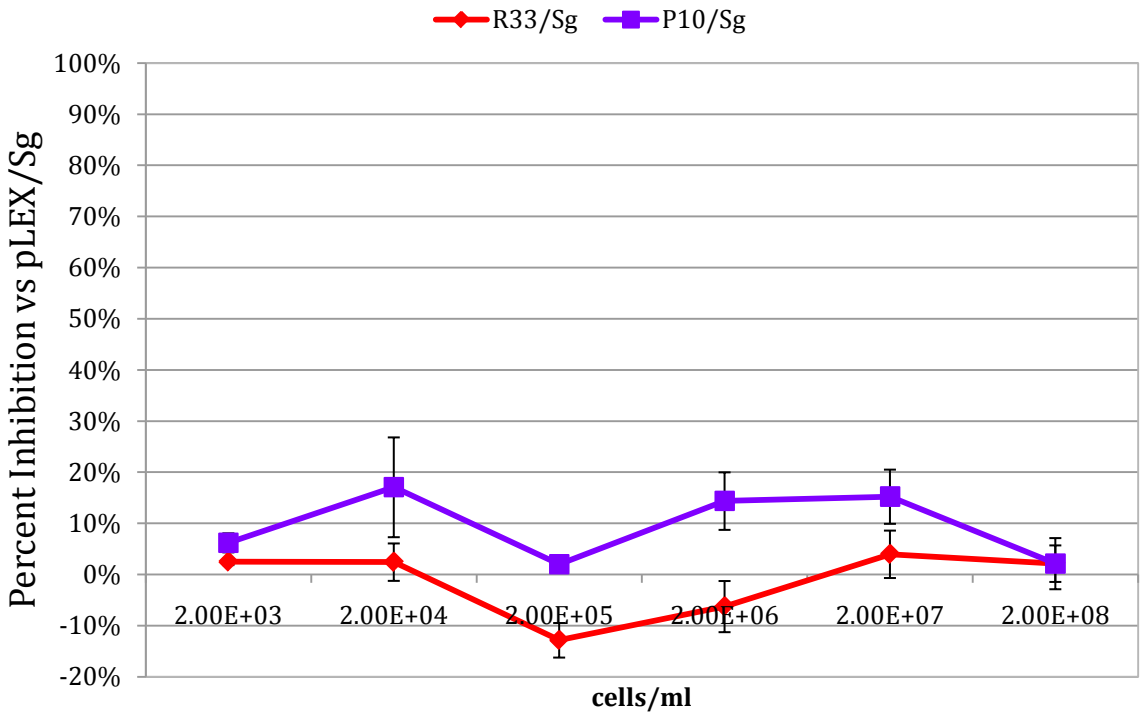


Table 2.1: Testing VHH in Vaginal HSV-2 Animal Challenge Model

CF-1 female mice were treated with Depo-provera, and one week after 10 ID_{50} HSV-2 G was mixed with A) PBS, P10, R33 or B) pLEX/Sg, P10/Sg, or R33/Sg and promptly delivered to the mouse vagina. Three days later the vagina was lavaged, and fluid was plated on foreskin cells to assay for the presence of virus.

A)

Sample	**Input**	**# Infected**	**Total #**	**% Infected**
Virus/PBS	10 ID_{50}	17	24	71%
P10	100 uM	10	17	59%
R33	100 uM	11	18	61%

B)

Sample	**Input (cells)**	**# Infected**	**Total #**	**% Infected**
pLEX/Sg	2 x 10^6	5	10	50%
P10/Sg	2 x 10^6	4	10	40%
R33/Sg	2 x 10^6	6	10	60%

Chapter Three: Expression of Anti-gD2 VHH With the Herpes Simplex Virus-2 Antimicrobial Peptide TATC Increases Antiviral Activity

Introduction

Herpes simplex virus-2 (HSV-2), one of the most prevalent sexually transmitted infections (STIs) in the world, infects approximately 16% of people ages 15-49[8]. While generally not life threatening, HSV-2 can have severe sequelae in immunocompromised individuals and in infants[156]. Additionally, HSV-2 infection is associated with a significantly increased risk of acquisition of human immunodeficiency virus-1 (HIV-1)[23, 24]. Complicating the effort to prevent transmission, a large proportion of HSV-2 primary infections and reactivations are subclinical, so asymptomatic individuals may still transmit the virus[11, 157]. Vaccine strategies to prevent HSV-2 transmission have not been broadly protective, and condoms are not always effective at preventing transmission[100, 101]. These failures have generated renewed interest in alternative prevention strategies, including the development of an effective microbicide to prevent transmission of HSV-2.

In the search for novel ways to prevent and treat HSV-2 infections, numerous antivirals and antimicrobial peptides (AMPs) with great potential as microbicides have been discovered[120]. AMPs are short (12 to 100 amino acid) positively charged, amphiphilic peptides that are typically part of an organism's innate immune defenses[158, 159]. Several anti-HSV AMPs have been demonstrated to have potent effects on various steps of the virus life cycle, and often also on disparate virus families[120, 159]. For example, the C5A peptide from hepatitis C virus (HCV) has

potent antiviral activity against HSV-2, HCV, and HIV[121]; the EB peptide, derived from the fibroblast growth factor 4 signal peptide, has activity against HSV and influenza[160, 161]; and lactoferrin has inhibitory activity against human papillomavirus (HPV) and HSV[162]. While the broad antiviral activity of these peptides is initially an attractive feature, it suggests a non-specific antiviral mechanism. Since the exact anti-viral mechanism of many HSV AMPs is poorly understood, this raises the possibility of unintended toxicity in host cells. Improving specificity and thereby reducing potential toxicity will be important for any microbicide development that employs AMPs.

Many research fields have exploited the specificity of antibody molecules to direct more toxic molecules to specific targets, such as cancer cells, bacteria, and viruses. For example, immunotoxins, in which part of exotoxin A from *Pseudomonas aeruginosa* is expressed with a cancer antigen-specific antibody, have been very successful at selectively killing cancerous cells and several immunotoxin candidates have progressed to human clinical trials[163]. The scaled up production of monoclonal antibodies and derivatives, such as single chain variable fragments (scFvs), for pharmaceutical application remains, however, a complex and expensive endeavor. Single domain antibodies, termed VHH, derived from the variable domain of heavy chain only antibodies found in members of the camelid family, offer an attractive method to easily conjugate and specifically deliver an AMP to the intended target. Combinations of llama VHH antibodies and AMPs have been attempted before, including the expression of a VHH against *Streptococcus mutans* fused to the synthetic AMP called dhvar5[164], although expression problems prevented successful testing of the VHH-AMP fusion protein to evaluate the efficacy.

One of the more promising HSV AMPs is derived from the cell-penetrating peptide (CPP) sequence of the HIV-1 Tat protein (amino acids 48-57). Although the CPP sequence from Tat is best known for its ability to transport heterologous proteins across the cell membrane[165], the Tat peptide (48-57) also has potent antiviral affects against HSV-1 and HSV-2 by preventing the virus from entering cells[160]. The addition of a single cysteine at the C-terminus to the peptide, called TATC, improves the efficacy of the peptide by conferring additional antiviral properties. In addition to acting as a fusion inhibitor to prevent virus entry, TATC was found to be able to directly inactivate virions and to induce a state of resistance to HSV infection when cells are pretreated with TATC[166]. In mice, treatment with the TATC peptide was found to significantly reduce the severity of kerititis in an ocular challenge model with HSV-1[167]. We hypothesize that expression of an anti-gD2 VHH fused to the TATC peptide would have improved *in vitro* antiviral activity compared to a non-specific VHH fused to TATC or the TATC peptide alone.

Materials and Methods

Cloning and Expression of VHH and VHHTATC in E. coli

A VHH that binds to gD2 of HSV-2 (called R33) was identified through the methods described in Chapter 2 Materials and Methods. P10, a VHH that does not bind to gD2 was also identified. VHH sequences were amplified using primers that introduced EcoRI and XhoI restriction sites for cloning in to pET-47b (Novagen Inc., Madison, WI). For VHHTATC constructs, oligos coding for the TATC sequence were annealed for insertion to the 3' end of the VHH sequence. The VHH and VHHTATC constructs were transformed in to BL21 DE3 competent cells (New England Biolabs, Ipswich, MA). For expression, an overnight (ON) 10 mL culture was diluted into 750 mL of Luria Broth (LB) / kanamycin (Kan) and grown until OD_{600} 0.6-0.8. After induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM for 3hrs at 37°C, cells were harvested by centrifugation at 3500g for 30 minutes, resuspended in 10 ml lysis buffer (6M Guanidine hydochloride, 0.1 M NaH_2PO_4, 0.01 M Tris base, 0.01 M imidazole, pH 8) and frozen at -80°C for at least 30 minutes. Upon thawing, the volume of the lysate was brought to 30 mL with lysis buffer, incubated with rocking at room temperature (RT) for at least 30 minutes, and then centrifuged at 14000 rpm for 30 minutes. After the pellet was discarded, Ni-NTA Agarose (QIAGEN, Valencia, CA) was added to lysate and rocked at RT for 1 hr or ON at 4°C. Beads were washed twice with 7 mL Wash Buffer 1 (8 M urea, 0.1 M NaH_2PO_4, 0.15 M NaCl, 0.02 M imidazole, pH 8) and then washed with ~50 mL (7 x 7 mL) Wash Buffer 2 (0.05 M NaH_2PO_4, 0.5 M NaCl, 0.02 M imidazole, pH 8). To elute VHH from beads, 4 x 1 mL Elution Buffer (0.05 M NaH_2PO_4, 0.5 M NaCl, 0.25 M imidazole, pH 8) was added for 1 hr at RT and collected. Eluted VHH were dialyzed

against PBS with 1 mM DTT with at least 4 buffer changes. The proteins were concentrated with Amicon Ultra-15 Centrifugal Filter Unit (EMD Millipore, Billerica, MA), centrifuged at 16000 g for 10 minutes to remove precipitated protein, and protein concentration was measured by Bradford assay (BioRad, Hercules, CA). Purified proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) for visualization with Coomassie Brilliant Blue staining.

Synthesis of TATC Peptide

The TATC peptide (GRKKRRQRRRC) was synthesized by GenScript, Inc (Piscataway, NJ) with greater than 95% purity. Peptide was resuspended in water at a concentration of 4 mg/ml.

ELISA to Validate VHH and VHHTATC Binding to gD2

An ELISA was performed to determine if purified VHH and VHHTATC bind to gD2. Wells of NUNC Maxisorp ELISA plates (Thermo Fisher Scientific Inc., Waltham, MA) were coated with various dilutions of VHH and VHHTATC made in PBS and incubated ON at 4°C. Purified gD2 (from *Pichia pastoris*, described in Ch 2) diluted in PBS-Tween 0.2% (PBS-T) was added to wells for 1hr at RT. Wells were washed 4 x 200µL PBS-T and the anti-gD antibody DL6 (Santa Cruz Biotechnology, Dallas, TX) diluted in PBS-T was added to detect gD2 binding by VHH. After a 1 hr incubation at RT, wells were washed again 5 x 200µL PBS-T and an anti-mouse secondary antibody conjugated to horse-radish peroxidase (HRP, Jackson ImmunoResearch, West Grove, PA) was added. After a final wash with 4 x 200µL PBS-T, 200 µL ABTS® ELISA HRP Substrate (KPL,

Gaithersburg, MD) was added per well. The plate was read at 405nm using a BioTek Synergy HT Plate Reader (Winooski, VT).

Toxicity Assay (MTS Assay)

The CellTiter 96® AQueous One Solution Cell Proliferation Assay (also called MTS assay) was used to determine toxicity of VHHTATC on cell lines, and the assay was carried out using the protocol recommended by the manufacturer (Promega, Madison, WI). Vero cells were plated in 96-well trays $4x10^6$ cells/tray overnight. The following day, dilutions of the VHHTATC proteins were added to wells and incubated overnight. About 16 hrs after the addition of protein, 20 µl of the CellTiter 96® AQueous One Solution reagent was added to each well and incubated 3 hrs at 37°C . The plate was read at 405nm with a BioTek Synergy HT Plate Reader (Winooski, VT).

Comprehensive Antiviral Activity of VHHTATC

Vero cells (ATCC CCL-81, Manassas, VA) were plated in Falcon 12-well trays (Thermo Fisher Scientific Inc., Waltham, MA) at 4 x 10^6 cells per tray and incubated ON at 37°C. Both the cells and the virus (at $5x10^3$ pfu/ml, HSV-2 G, ATCC VR-734, Manassas, VA) were incubated with dilutions of the TATC, VHH, or VHHTATC protein for 1 hr at 37°C. After the 1hr incubation, the protein-treated virus was added to the cells for a 1 hr adsorption period with gentle shaking every 10 minutes to ensure even distribution of the virus over the cells. Cells were overlaid with 1 mL of 0.5% methylcellulose/5% FBS, incubated for 2 days at 37°C, and stained with crystal violet for counting plaques.

Flow Cytometry to Detect VHH and VHHTATC Binding to Vero Cells

Nearly confluent Vero cells (ATCC CCL-81, Manassas, VA) were trypsinized, washed once with PBS, and resuspended at 0.5 x10^6 cells/ml. 500 µL of the cell suspension was aliquoted, centrifuged at 500 g for 5 minutes, and resuspended with 10 ug/ml R33, P10, R33TATC, P10TATC, or DL6 in PBS in 100 µL and incubated at 37°C for 1 hr. Cells were washed once with PBS and resuspended in 1 mL 1% BSA/PBS and incubated at 37°C for 30 minutes for blocking. Cells were washed twice with 2 mL PBS and resuspended in anti-His antibody at 1:1000 for 30 minutes at 4°C. Cells were washed twice with 2 mL PBS and resuspended in fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) at 1:500 diluted in 1% BSA/PBS for 30 minutes at 4°C, followed by a final wash with 2 mL PBS. Samples were run on a Becton-Dickinson FACSCalibur Cytometer and data were analyzed using FloJo (Tree Star Inc., Ashland, OR).

Statistical Analysis

For the comprehensive antiviral assay, the significance of the difference in plaque numbers was calculated using an ANOVA test, with a Bonferroni correction (STATA Corp, College Station, MD).

Results

Expression and Purification of VHHTATC

The gD2-specific VHH, R33, and a non gD2-binding VHH, P10, were identified and cloned as described in Chapter 2 Materials and Methods. For VHHTATC (R33TATC and P10TATC), the TATC sequence (GRKKRRQRRRC) was inserted at the C-terminus of the R33 and P10 sequences already cloned into the pET-47b expression vector. The VHH/pET and VHHTATC/pET constructs were transformed into BL21 DE3 competent cells and expressed as previously described. Expression of VHH with TATC resulted in induced proteins only being present in the insoluble fraction, so all VHH and VHHTATC were purified using the insoluble protein purification protocol described in the Materials and Methods.

E*LISA to Determine VHHTATC Binding to gD2*

To verify that expression of VHH with TATC did not change the binding capability of the VHH, an ELISA was performed comparing the gD2-binding activity of VHH alone (R33 and P10) to VHHTATC (R33TATC and P10TATC) and the TATC peptide alone. Figure 3.2 demonstrates that the addition of the TATC did not affect the ability of R33 to bind to gD2, as R33 and R33TATC had similar levels of reactivity to gD2. Additionally, the TATC peptide did not confer any reactivity to gD2 as P10TATC and the TATC peptide alone did not bind to gD2.

Toxicity of VHHTATC on Vero Cells

With the MTS assay, a reagent is added to cells that is processed by living cells and generates a byproduct that can be measured at OD_{490}, thereby serving as a way to measure how toxic a treatment is to cells. Previous reports on the toxicity of the TATC peptide showed that the peptide was not toxic up to 400 μM as measured by an MTS assay[166]. Although molarities that high for VHHTATC could not be tested due to protein precipitation at high concentrations, it was determined that R33TATC, P10TATC, and the TATC peptide did not induce cytotoxicity in Vero cells for a range of approximately 10μM to 10^{-4}μM (Figure 3.3).

Comprehensive Antiviral Assay

A comprehensive antiviral assay, in which both the cells and the virus are pretreated with VHHTATC, VHH, or TATC and then the treated virus is added to cells so that the proteins are also present during the entry step, was performed to determine if the addition of a gD2-specific VHH (R33) to the TATC sequence could enhance its antiviral activity. Figure 3.4 is a representative experiment that shows that the antiviral activity exhibited by all proteins that include TATC are significantly different than R33 at the highest protein concentration tested (70μM). At this concentration there is no significant difference in the antiviral activity between the VHHTATC (R33TATC or P10TATC) and TATC alone. However, upon further dilution of the proteins, R33TATC maintains its antiviral activity while P10TATC acts similar to the TATC peptide and no longer has antiviral activity. For R33TATC, significant antiviral activity is observed for

dilutions ranging from 3.5μM to $4x10^{-4}$ μM compared to R33, untreated virus, TATC, and P10TATC (Figure 3.4).

Binding of VHHTATC to Vero Cells

To explore how pretreating cells with VHHTATC might be able to inhibit HSV-2 infection, flow cytometry was performed to see if these proteins could bind to Vero cells. Vero cells were trypsinized, washed, and incubated with VHH and VHHTATC for 1 hr at 37°C, as they are in the comprehensive antiviral assay. Binding of these proteins was then detected using staining with an anti-His antibody, followed by a FITC-conjugated secondary antibody. Because R33 is specific for gD2, neither R33 nor P10 should be able to bind to uninfected Vero cells, and this was confirmed by FACS (Figure 3.5). When Vero cells were incubated with VHHTATC proteins, however, R33TATC and P10TATC are detected binding to the surface of unpermeablized Vero cells (Figure 3.5). Since the only difference between the VHH and VHHTATC is the presence of the C-terminal TATC peptide, it suggests that the TATC peptide is the factor allowing for these proteins to nonspecifically bind to the cell surface.

Discussion

A number of antimicrobial peptides from diverse origins with activity against HSV-2 have been reported as potential microbicide candidates[121, 123, 160, 168]. Very little is known about the exact mechanism of these AMPs, however, including the AMP included in this study, TATC. The TATC peptide is derived from the cell-penetrating peptide sequence of the HIV-1 Tat protein. TATC is reported to have antiviral activity against HSV-2 at three points in the viral entry process: induction of a state of resistance to viral infection in pretreated cells, virucidal activity, and fusion inhibitor at entry[160, 166]. Given the potential for off-target toxicity with such broad-spectrum activity, a gD2-specific VHH (R33) and an irrelevant VHH (P10) were expressed with a C-terminal TATC to increase the antiviral activity and specificity of the TATC peptide. The antiviral activity of R33TATC was compared to P10TATC, R33, and the TATC peptide in an antiviral assay in which both the cells and the virus were pretreated with the proteins. P10TATC had antiviral activity equivalent to the TATC peptide, indicating that expression with a VHH was not affecting the antiviral activity of TATC. R33TATC exhibited a statistically significant increase in virus inhibition compared to untreated virus and R33 at all but the lowest concentrations tested, and against TATC and P10TATC in the range of 3.5 to 4 x 10^{-4} μM (Figure 3.4). Although the IC_{50} value of the R33TATC (approximately 1-5μM) is not particularly noteworthy compared to other AMPs, this is the first time it has been demonstrated that expression of an AMP as a fusion protein with a VHH can improve the activity of the AMP.

The curve of virus inhibition formed by the R33TATC data points is somewhat unusual in that there is a plateau of virus inhibitory activity between approximately 0.1

and 0.001 μM. Despite multiple repetitions of this experiment and the addition of more data points in this range (data not shown), the plateau persisted. There is a change in the slope of the virus inhibitory activity starting at around 0.1μM, indicating that the activity of R33TATC is not following a simple dose-response curve. One potential hypothesis is that at higher concentrations, the amount of TATC present is sufficient to inhibit the virus independent of any specificity provided by R33, but at lower concentrations the R33 portion of the R33TATC protein is able to direct the AMP to gD2 where it can interfere with the entry process with greater efficiency. The experiments completed in this chapter were not able to address this proposed hypothesis, and additional experiments would need to be undertaken to explore the kinetics of R33TATC antiviral activity.

Although TATC is able to intervene at various points in the HSV-2 entry process, the antiviral effect of TATC at each of these points is not equal. For example, the reported direct virucidal effect of the TATC peptide is quite minimal, with an IC_{50} of 96-110 μM[166]. The TATC peptide is most effective at preventing infection when cells are pretreated with the peptide prior to addition of virus (IC_{50} of 3.3-4.5 μM) or when the TATC peptide is present during the entry step (IC_{50} of 3.1)[19]. From previous publications, however, there is no proposed mechanism as to how this might be occurring, so it is not clear how the TATC peptide is acting during the pretreatment of cells to prevent infection, and in the case of the antiviral activity of R33TATC, how the gD2-specific activity of R33 might be enhancing the antiviral activity. Given that the TATC peptide contains the protein transduction domain of the Tat protein, and therefore can cross cellular membranes, one possibility is that the TATC peptide is embedding in the cellular membrane, where it is able to act as a fusion inhibitor when the virus contacts the cell

surface. To determine if the TATC peptide was conferring the ability of VHHTATC to non-specifically bind to or embed in cell membranes, FACS was performed with Vero cells that had been incubated with VHH and VHHTATC, as they are in the comprehensive antiviral assay. The results demonstrated that the presence of the TATC peptide does indeed allow for R33TATC and P10TATC to bind to Vero cells, indicating a nonspecific binding of these proteins to the cell membrane. The cells were not permeablized so the binding that was detected was occurring at the cell surface.

This intriguing finding leads to the hypothesis that the TATC portion of R33TATC embeds in the membrane so that R33 decorates the surface of uninfected Vero cells. When the virus comes in contact with the cellular membrane, the gD2 binding activity of R33 may bind the virus and prevent it from interacting with the cellular proteins necessary for entry. To test this hypothesis, a cell line expressing R33 at the surface would be expected to have similar effects on preventing virus entry. Alternatively, gD2 could be added to R33TATC-treated cells prior to virus infection so that the R33 binding sites are occupied, leaving the virus free to bind to its receptors and enter the cell.

While it may be interesting to further explore the mechanism of the antiviral activity of TATC and how R33 can improve its function, the main importance of this research is that it demonstrates that a VHH can be used to enhance the antiviral function of an attached antimicrobial peptide. A previous similar attempt in which a synthetically derived AMP was expressed with VHH lost all activity when expressed as a fusion protein[169]. Other HSV-2 antimicrobial peptides may be better candidates for this strategy of increasing antiviral activity, and is also possible that other enveloped viruses

may be more susceptible to the effects of a VHHTATC construct. Given that in an *in vivo* setting the concentration of any microbicide is likely to not reach the maximal levels tested *in vitro*, this may be an important way to make an AMP biologically relevant.

Figures

Figure 3.1: Purification of VHH and VHHTATC

VHH and VHHTATC were expressed and purified from *E. coli*, and 800 ng of each VHH sample was separated by SDS-PAGE to evaluate size and purity.

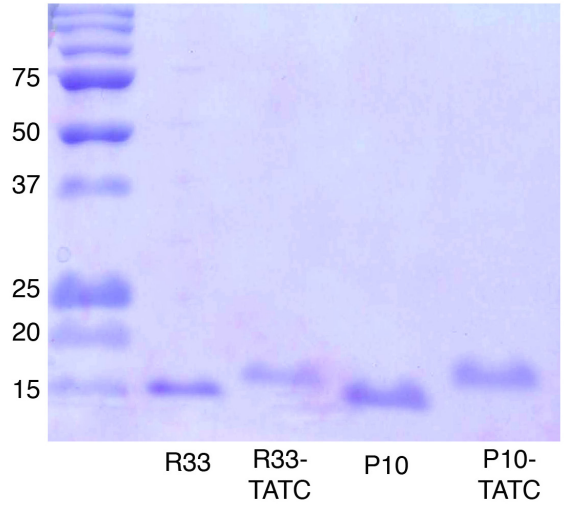


Figure 3.2: VHH and VHHTATC Binding gD2

An ELISA was performed in which wells were coated with the VHH, VHHTATC, and TATC. After gD2 was added to the wells, binding was detected with a gD2-specific antibody (DL6). Each dilution was assayed in duplicate and error bars represent maximum and minimum values.

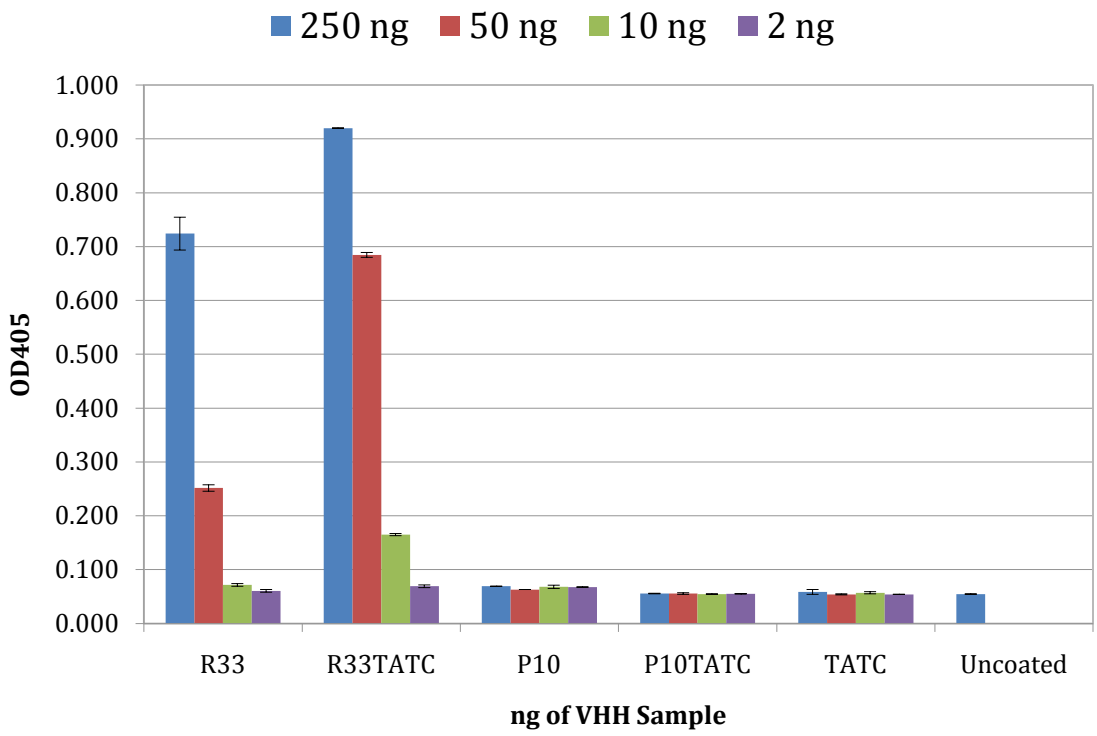


Figure 3.3: Toxicity of VHHTATC and TATC on Vero Cells

The toxicity of the VHHTATC proteins on Vero cells was compared to TATC and media alone. Dilutions of each protein were added to wells in triplicate, incubated overnight, and after addition of MTS reagent for 4 hours, the plate was read at OD_{490}. Higher OD_{490} readings indicate lack of toxicity. Error bars represent standard deviation.

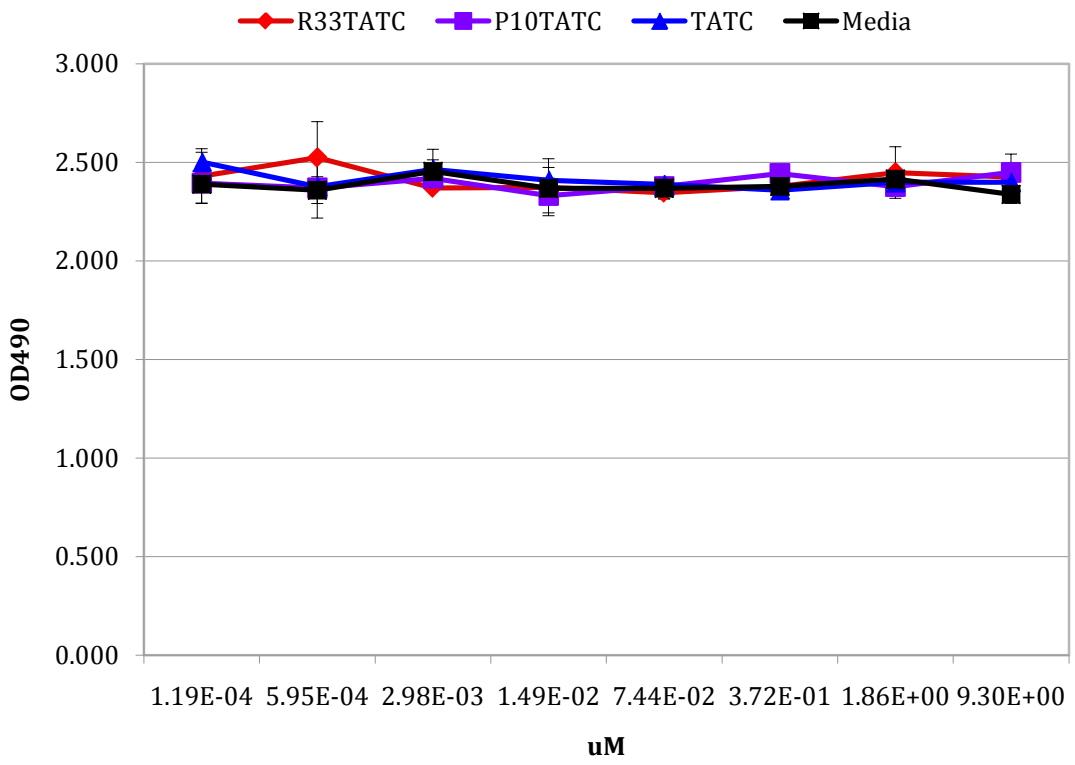


Figure 3.4: Comprehensive Antiviral Assay

Both cells and virus were pretreated with dilutions of VHHTATC, VHH, or TATC, and virus was added to cells to assay for antiviral activity. Each dilution was assayed in duplicate and error bars represent maximum and minimum plaque numbers. Asterisks indicate that data point is significantly different from untreated virus, R33, P10TATC, and TATC ($p<0.05$) as calculated by ANOVA.

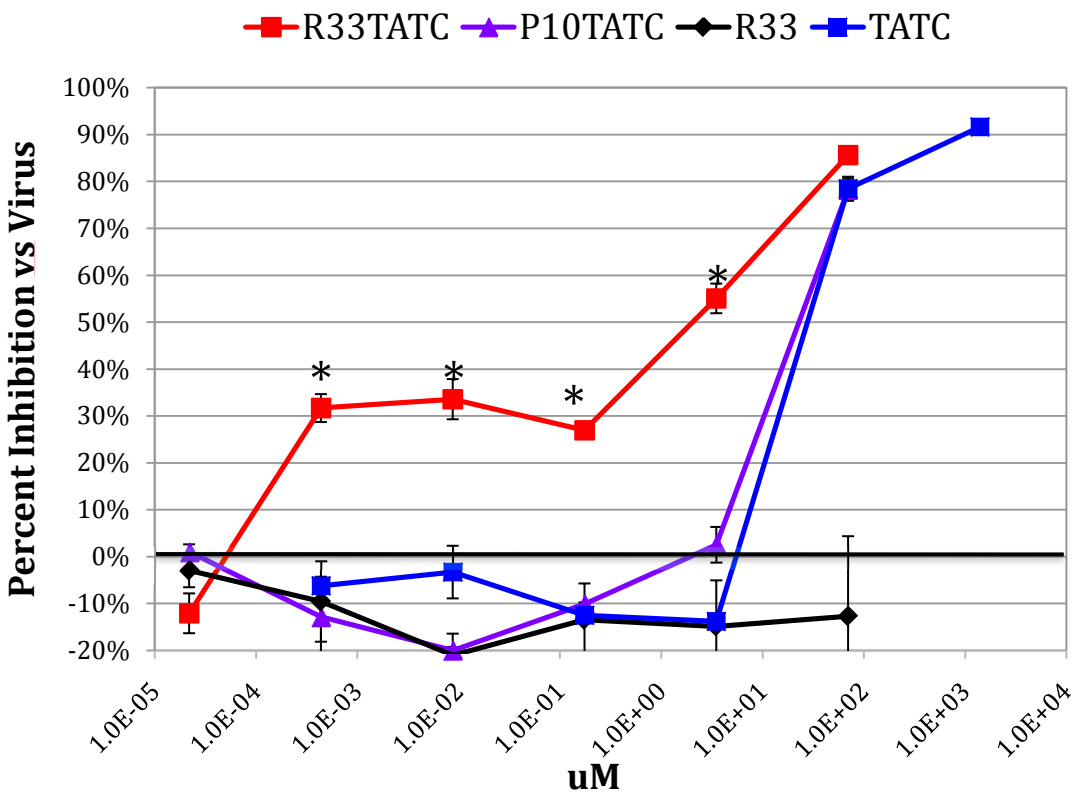
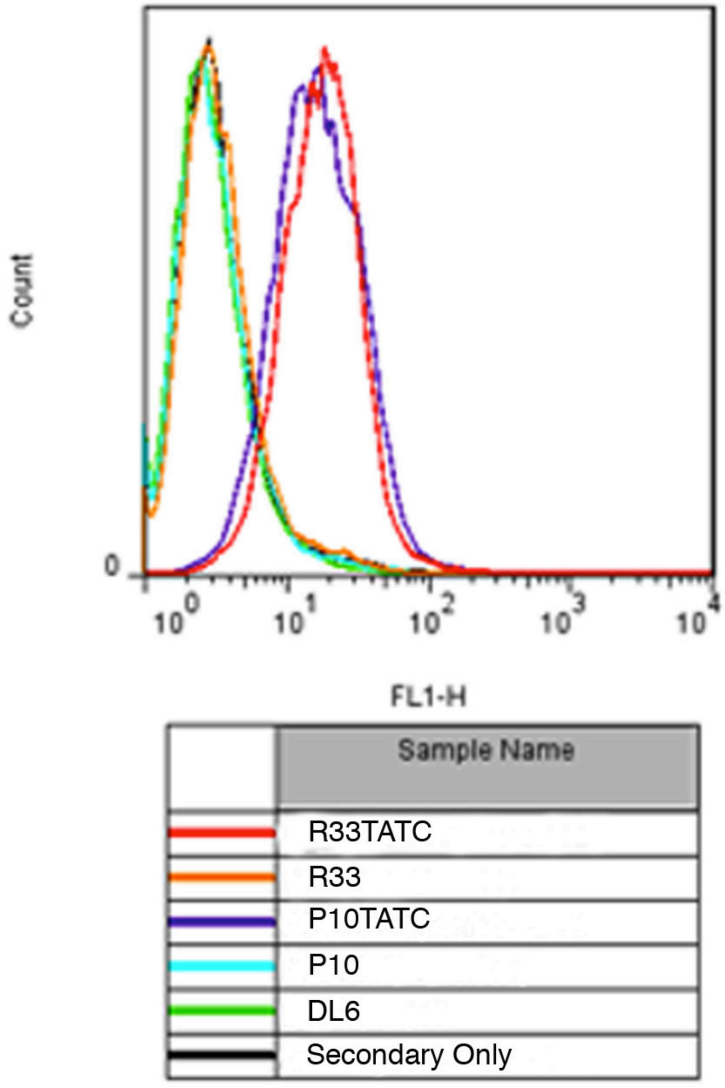


Figure 3.5: VHH and VHHTATC Binding to Vero Cells

Vero cells were incubated with VHH or VHHTATC proteins before staining with an anti-His antibody followed by anti-mouse secondary conjugated to FITC to detect VHH or VHHTATC binding. Both TATC-containing proteins (R33TATC and P10TATC) demonstrated binding at the cell surface, while R33, P10, DL6 (anti-gD antibody), and the secondary antibody had no reactivity to Vero cells.



Chapter Four: Efficacy of Single Domain Antibody Immunotoxin Binding to Glycoprotein D of Herpes Simplex Virus 2 to Prevent Infection *In Vitro* and *In Vivo*

Introduction

The variable domain of heavy-chain only antibodies found in members of the camelid family represents the smallest naturally occurring functional domain of the antibody molecule[139]. These variable domains, termed VHH, have the same antigen binding capability as full-length antibodies, yet are typically around 15 kDa in size. When cloned and purified as monomeric domains, VHH demonstrate remarkable stability under a wide range of denaturing, temperature, and pH conditions[140]. VHHs exhibit increased solubility compared to full-length antibodies or other antibody fragments, and very high expression levels have been achieved in *E. coli*, yeast, and tobacco expression systems[170-172]. Due to a high degree of sequence homology between camelid and human variable domains, VHH have been shown not to be immunogenic in mice[173]. As a result of their small size, VHH have enhanced tissue penetration[173], and an extended CDR3 loop allows VHH access to cryptic epitopes in enzymatically active sites that are unavailable for binding by full length antibodies[174, 175].

Given their unique combination of characteristics, VHH have been promoted as promising biomedical tools. A myriad of VHH have been successfully developed for diverse purposes including diagnostics[145, 176, 177], imaging[178, 179], and biochemical[180, 181] and therapeutic applications[133, 169, 182]. In terms of the diversity of pathogens that have been targeted thus far, VHH directed against viruses[133, 135, 141, 183], bacteria[175], protozoa[184], and fungi[185] have all been

identified. VHH can act as a monomeric domain, or they can be expressed in a multivalent context to increase avidity and activity[135, 147, 186]. Additionally, bispecific VHH can be assembled that bind different epitopes, which can in some cases dramatically increasing neutralization efficacy[133].

VHH can also serve as carriers for other molecules through conjugation or expression as a fusion protein with an effector domain. One of the most successful applications of this technology has been in the cancer field, where the small size and antigen specificity of the VHH is harnessed to precisely deliver a conjugated drug[187, 188], enzyme[189], or toxin[190] to cancerous cells. A substantial amount of work has been published describing the construction of an immunotoxin through expression of a cancer-specific single chain variable fragment (scFv) with the effector domains of exotoxin A from *Pseudomonas aeruginosa* (PE), and several PE-based immunotoxins have progressed to clinical trials[163]. While most PE-based immunotoxins use a scFv as the antibody portion, it was recently demonstrated that a VHH binding to vascular endothelial growth factor receptor 2 (VEGFR2) has been used as the antibody portion of an immunotoxin to target tumor cells[190].

Despite years of research investigating vaccines designed to prevent sexual transmission of herpes simplex virus-2 (HSV-2), there is still no protective vaccine against one of the most common sexually transmitted infections (STIs) in the world. In the search for a neutralizing VHH directed against glycoprotein D 2 (gD2) of HSV-2 that could serve as a microbicide to prevent transmission of this virus, we identified a VHH that specifically binds gD2. Although able to bind to gD2, the single domain VHH failed to neutralize the virus; therefore we sought to use the gD2 binding capability of this VHH

to create a PE-based immunotoxin (VHHExoA). Of relevance to HSV-2, Kaposi's sarcoma-associated herpesvirus (KSHV)-infected cells in the lytic stage of infection can be successfully killed by an immunotoxin directed against surface expressed-glycoprotein H[191]. The *in vitro* efficacy of this strategy indicates that surface viral glycoproteins are feasible targets for immunotoxin therapies. If applied vaginally, an anti-gD2 immunotoxin could potentially prevent HSV-2 infection by killing infected epithelial cells prior to establishment of latency. This immunotoxin has the potential to not only act as a microbicide to prevent initial infection, but could also act to reduce viral shedding in infected individuals by eliminating gD2-expressing cells during reactivation of the virus from latency.

Materials and Methods

Expression, Purification, Refolding of VHHExoA

A VHH that binds to gD2 of HSV-2 (called R33) was identified through the methods described in Chapter 2 Materials and Methods. P10, a VHH that does not bind to gD2 was also identified. VHH sequences were amplified using primers that introduced EcoRI and XhoI restriction sites for cloning in to pET-47b (Novagen Inc., Madison, WI). The previously published exotoxin A sequence[192] was synthesized (GenScript, Inc, Piscataway, NJ) and cloned in frame to the C-terminus of the VHH (R33 and P10) already present in the pET-47b vector. Expression, purification, and refolding of VHHExoA proteins were performed based on a previously published protocol[193]. Briefly, large-scale cultures (800 mL) of transformed BL21 DE3 cells (New England Biolabs, Ipswich, MA) were grown to OD_{600} 0.6 and induced with 1 mM with IPTG (Lab Scientific, Inc, Highlands, NJ) for 3 hrs at 37°C. After cells were harvested and lysed under denaturing conditions (6 M GuHCl), Ni-NTA Agarose (QIAGEN, Valencia, CA) was added to the clarified lysate to purify the His-tagged VHHExoA protein. After washing, VHHExoA protein was eluted (8 M urea, 250 mM imidazole, 50 mM NaH_2PO4, 500 mM NaCl, 300 mM DTT) diluted 1:100 in refolding buffer (100 mM Tris, 500 mM L-arginine, 8 mM oxidized glutathione, 2 mM EDTA), and incubated at 10°C overnight. After completion of the refolding reaction, the refolded VHHExoA was concentrated with an Amicon Ultra-15 Centrifugal Filter Unit (EMD Millipore, Billerica, MA) and buffer exchange was performed by repeatedly bringing up the volume of the concentrated protein with PBS. The final volume of the protein was brought to ~1mL,

aliquoted, and frozen at -80°C until use. Protein concentration was determined using a Bradford assay (BioRad, Hercules, CA).

Toxicity Assay (MTS Assay)

The CellTiter 96® AQueous One Solution Cell Proliferation Assay was used to determine toxicity of VHHExoA on cell lines, and the assay was carried out using the protocol recommended by the manufacturer (Promega, Madison, WI). Z4/6 cells[148], expressing gD2 at the cell surface, and the parental L cell line (ATCC CRL-2648, Manassas, VA) were plated in 96-well trays at 3 x 10^5 cells/well overnight. The following day, dilutions of the VHHExoA proteins were added to wells and incubated overnight. About 16 hrs after the addition of protein, 20 µL of the CellTiter 96® AQueous One Solution reagent was added to each well and incubated 4 hrs at 37C. The plate was read at 490 nm with a BioTek Synergy HT Plate Reader (Winooski, VT). Higher OD values indicate greater cell viability, and therefore, less toxicity.

ELISA: Binding of VHHExoA to gD2

An ELISA was performed to determine if the purified VHHExoA was capable of binding to gD2. NUNC ELISA plates were coated with dilutions of VHHExoA (0.25 µg/well), and after a blocking step, dilutions of purified gD2 were added to wells in duplicate. After a washing step of 5 x 200 µL PBS-0.05% Tween (PBS-T) wash buffer, the anti-gD antibody DL6 (Santa Cruz Biotechnology, Dallas, TX) was added at 1:1000 for 1 hr. Wells were washed again and HRP-conjugated anti-mouse (Sigma-Aldrich, St. Louis, MO) was added at 1:3000 for 1 hr. A final wash step was performed, and plate was

developed by adding 100 µl/well ABTS® ELISA HRP Substrate (KPL, Gaithersburg, MD). The plate was read at 405nm using a BioTek Synergy HT Plate Reader (Winooski, VT).

In Vitro Infectious Center Assay (ICA)

Vero cells were plated in 12-well trays at 4 x 10^6 cells/tray and after 24 hours were infected with HSV-2 G (ATCC VR-734, Manassas, VA) at 500 pfu/well. Following the 1 hr adsorption time, dilutions of the VHHExoA proteins were added to wells in duplicate and complete media (DMEM, CellGro, Manassas, VA) was added to bring volume up to 700 µL per well. About 16 hrs later, supernatant was removed and cells were trypsinized briefly with 250 µL trypsin/EDTA (CellGro, Manassas, VA) before adding an equal volume of complete media. Cells were centrifuged at 500 g for 5 minutes to pellet cells, and then resuspended in 200 µL of complete media. Dilutions of the infected Vero cells were made in uninfected Vero cells harvested the same day, and plated in 12-well trays so that total cell number was roughly 3 x 10^5 cells/well. Cells were overlaid with 0.5% methylcellulose/5% FBS to bring volume to 1ml. After 2 days, cells were stained with crystal violet and plaques were counted.

Animal Experiments

Six to eight week old female CF-1 mice were purchased from Harlan (Indianapolis, IN) and housed under reversed photoperiod conditions. As reported previously[152], on Day 0 mice are injected subcutaneously in the hind quarters with 2.5 mg of Depo Provera (UpJohn Co. 400 mg/ml) one week before the planned viral challenge. On Day 7, 10 µL

of the virus inoculum (10 ID_{50}) is combined with 10µL of the VHHExoA (20 µM, therefore final concentration is 10µM) and 20 µL is promptly delivered to the vagina with a fire-polished Wiretrol pipet (Drummond Co., Broomall, PA). Six, 24, and 48 hours post challenge mice are vaginally treated with a 10 µL of 20 µM dose of VHHExoA. On Day 10 the vagina is lavaged using 20 µl of Bartel's Tissue Culture Refeeding Media; the fluid is delivered vaginally and withdrawn 10 to 20 times to collect HSV shed into the vagina. The lavage fluid is centrifuged at 6500 rpm for 5 minutes to remove mucus and cells, and then placed on human newborn foreskin cells to assay for presence of virus. Cells are observed by microscope 48 hours later (Day 13) and scored yes/no for infection. All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University (Protocol Number MO12H147).

Statistical Analysis

For the ICA, the significance of the difference in plaque numbers was calculated using an ANOVA test, with a Bonferroni correction (STATA Corp, College Station, MD). For analysis of the results from animal HSV-2 challenge experiments, a Fisher exact test (two-tailed) was used to compare the number of animals infected between experimental and the control groups.

Results

Purified R33ExoA Binds to gD2

Purification of immunotoxins is potentially complicated by the need to optimize conditions so that the antibody and exotoxin components of the molecule will each fold correctly and maintain their distinct functions. The VHHExoA immunotoxins were purified from induced BL21 cells (Figure 4.1). Based on protocols published by Buchner et al for an scFv immunotoxin[193], the VHHExoA (R33ExoA and P10ExoA) were refolded and the antibody function was tested by ELISA. R33ExoA was still able to bind gD2 at levels comparable to R33 alone, while P10 and P10ExoA had no gD2 binding activity (Figure 4.2).

Purified R33ExoA is Functional as an Immunotoxin

We next tested the exotoxin capability of the immuntoxin. To do this, we used a MTS assay with z4/6 cells that express gD2, and the parental cell line, L cells, that do not express gD2. Z4/6 cells are known to not have uniform expression of gD2[148], as shown in Figure 2.14 through staining with the anti-gD antibody DL6. The levels of gD2 expression, however, should be sufficient to determine if R33ExoA can exert a cytotoxic effect on cells expressing gD2. Figure 4.3 demonstrates that R33ExoA only has a cytotoxic effect on z4/6 cells and not the parental cell line, and that the non-gD2 binding P10ExoA has no cytotoxic effect on either cell line compared to cells treated with media alone.

Infectious Center Assay (ICA)

To test the cytotoxic effect of the R33ExoA on HSV-2 infected cells, an infectious center assay was performed (Figure 4.4). Cells were infected with HSV-2 in the presence of the VHHExoA, and allowed to proceed for roughly 16 hrs. Cells were harvested at this time, mixed with uninfected target cells, and then diluted and plated so that the number of infectious centers can be quantified. R33ExoA consistently demonstrated potent antiviral activity compared to P10ExoA and R33 with no ExoA, which had no antiviral activity. Multiple repetitions of the ICA revealed that the IC_{50} of R33ExoA is between 0.01 and 0.1 μM.

R33ExoA Activity as a Microbicide

The VHHExoA immunotoxins were tested in the mouse microbicide model to determine if they have the ability to protect against vaginal HSV-2 infection. Three treatments were used: PBS, R33ExoA, and P10ExoA. The viral inoculum was first mixed with VHHExoA (infection control group was mixed with PBS) before introduction in to the vagina. At three time points post-infection (6, 24, and 48 hrs) animals received additional doses of VHHExoA proteins (or PBS) so that the immunotoxins were present in the vagina at times when gD2 would be expressed at the cell surface and to mimic the repeated application that would accompany its use as a microbicide or treatment in humans. The infection control group (received only PBS) was infected at a frequency of 89% (8 out of 9 animals), indicating the virus was properly infectious. Similarly, in the group of animals that received P10ExoA, 7 out of 8, or 88% of animals became infected. Only 3 out of 8, or 38%, of animals treated with R33ExoA became infected, a level that

is statistically significant when compared to the infection control group (P = 0.048), but does not reach statistical significance when compared to the P10ExoA group (P = 0.119). Nonetheless, this result is promising and indicates that further animal testing exploring the use of R33ExoA as a microbicide or treatment is warranted.

Discussion

Using a VHH antibody that binds to gD2 of HSV-2, we created a PE-based immunotoxin that specifically targets HSV-2 infected cells. This immunotoxin was designed to bind to cells expressing gD2 at the cell surface, causing internalization of the entire protein, allowing the exotoxin A portion to act by halting protein synthesis, ultimately resulting in cell death[194]. Expression of R33 with exotoxin A did not inhibit the ability of R33 to bind to gD2, and did not impart any gD2 reactivity to the negative control, P10ExoA, as measured by ELISA (Figure 4.2). The immunotoxin was functional based on specific cytotoxicity on cells expressing gD2 compared to the parental cell line that does not express gD2 (Figure 4.3). When tested in an infectious center assay to determine activity against HSV2-infected cells, R33ExoA exhibited potent antiviral effects with an IC_{50} of between 0.01 and 0.1 μM based on four repetitions, and this activity was specific to expression of gD2 since P10ExoA had no activity in the range of molarities tested (Figure 4.4).

Due to the lack of toxicity on uninfected cells and the potent, specific activity on virus-infected cells, R33ExoA was a suitable candidate for further testing in a mouse HSV-2 microbicide model. The results from the mouse HSV-2 challenge model suggest that R33ExoA has potential as a microbicide for protection from acquisition of HSV-2 infection (Table 4.1), and additional experiments to verify this are underway. As a microbicide, if R33ExoA were present in the vagina at the time of viral challenge, the immunotoxin could preemptively kill virally infected epithelial cells prior to the spread of the virus to the nervous system. Although this was not tested in the animal model, in the case of a previously infected individual, R33ExoA could potentially also be used

therapeutically to treat viral reactivations and halt the shedding of infectious virus from infected epithelial cells that could be transmitted to an uninfected partner. Additionally, conceptually it is also possible that this immunotoxin could be used as a way to kill infected neurons, estimated to be a relatively low number (around 3%)[191], thereby eliminating the latent viral reservoir.

The majority of previous immunotoxin research has focused on targeting blood cancers, in which case the immunotoxin treatment is administered intravenously for systemic distribution to reach the cancer cells. Additionally, candidate immunotoxins directed against viruses such as HIV-1 or human cytomegalovirus would also require systemic treatment. The work described here represents the first immunotoxin designed for targeting a pathogen at a mucosal site of infection. Therefore, there are many unknown variables that could affect the efficacy of the R33ExoA immunotoxin when administered to the genital tract. The mouse model, while an important tool in evaluating microbicide or antiviral candidates, may not be representative of humans, particularly due to the differences in pH in the mouse and human vagina. Therefore, further *in vitro* studies evaluating the activity of R33ExoA under the lower pH conditions, for example, found in the human vagina should be undertaken. In this regard, having a VHH as the antibody portion of the immunotoxin is particularly advantageous due to its stability at a wide range of temperatures and pHs[195].

Another variable that should be explored further is how the kinetics of gD2 expression in an HSV-2 infection affect R33ExoA efficacy. The levels of gD2 expression *in vitro* are certainly different than what occurs *in vivo*, especially given whether it is an initial infection or a reactivation from latency. The infectious center assay was performed

when gD2 was abundantly expressed at the cell surface, and evaluating R33ExoA at other time points with differing levels of gD2 expression may be more informative regarding its activity during an active infection *in vivo*. The results of the animal experiments suggest that at the concentration of R33ExoA tested, the level of gD2 expression on epithelial cells may be high enough for R33ExoA to eliminate the infected cells prior to the establishment of a latent infection.

While it is significant that we have demonstrated that an anti-gD2 VHH can be used as the antibody portion of an immunotoxin to specifically target HSV-2 infected cells, the use of such an immunotoxin as a microbicide or antiviral treatment against HSV-2 must be applied with caution. Although the apoptosis that results from internalization of exotoxin A is generally a non-inflammatory process[196], the effect of vaginal treatment with R33ExoA must be further studied to determine if it could possibly lead to inflammation or the infiltration of immune cells, which could facilitate the transmission of other STIs, particularly HIV-1. Results from use of immunotoxins given systemically to treat cancer have demonstrated that PE-based immunotoxins can be immunogenic, and efforts are underway to reduce the immunogenicity of the exotoxin A portion of the protein[197]. The genital tract in general has a dampened immune response compared to other areas of the body[198], but the potential immunogenicity of exotoxin A is still a concern that must be evaluated. So while using HSV-2 immunotoxin as a microbicide may be effective in animal models of HSV-2 transmission, its use in humans may ultimately need to be restricted to low-risk individuals so that the potential increased inflammation and infiltration of immune cells does not lead to increased risk of HIV-1 or other STI acquisition. Ultimately the theoretical enhanced transmission risk associated

with killing of HSV-2 infected cells must be weighed against the enhanced HIV-1 transmission risk associated with HSV-2 infection.

A second concern regarding use of immunotoxins to target HSV-2 is that the virus establishes a latent infection in neurons, which are non-mitogenic cells that cannot be regenerated. So while it may be theoretically possible to eliminate latently infected neurons with this immunotoxin, this would come at the expense of loss of neurons in the genital tract. The penetrating capabilities of immunotoxins through the genital epithelium are unknown, so it is unclear if neuronal would could be a significant outcome of vaginal immunotoxin treatment. Additionally, the extent of gD2-expressing neurons in the vaginal tract is currently debated but is thought to be low[199], and it is possible that the benefit of the elimination of some latently infected neurons could outweigh the loss of those neurons. Given the progress that cancer immunotoxins have made in terms of efficacy and safety, there is reason for optimism that a similar strategy could be used to prevent or treat HVS-2 infections.

Figures and Tables

Figure 4.1: Purification of VHHExoA

VHHExoA were purified from the insoluble fraction of induced *E. coli* cells and refolded according to previously published protocols[193].

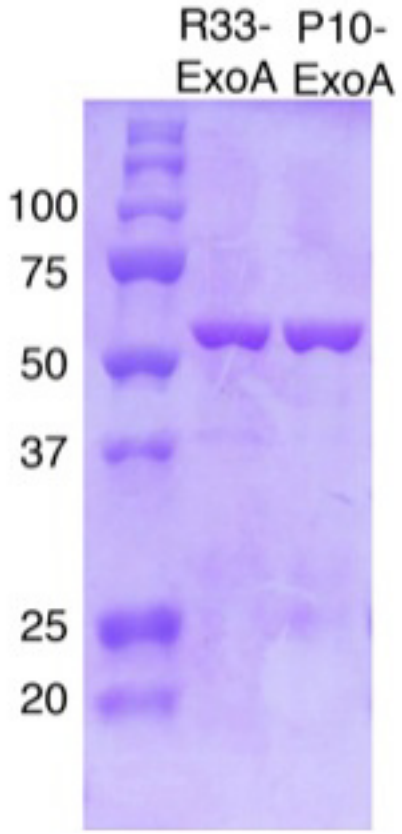


Figure 4.2: VHH and VHHExoA Bind to gD2

A capture ELISA was performed to determine if the VHH portion of R33ExoA is able to bind gD2 when expressed with a C-terminal exotoxin A. Each dilution was assayed in duplicate and error bars represent maximum and minimum values.

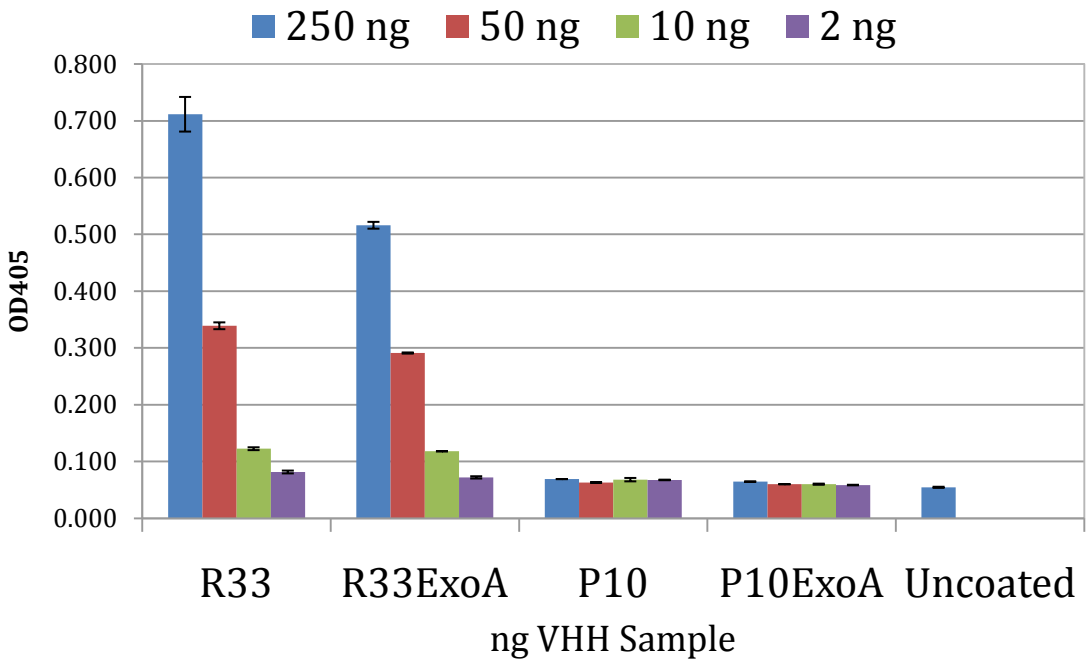


Figure 4.3.A: Toxicity of VHHExoA on Vero cells and z4/6 cells

A) Dilutions of VHH-ExoA proteins were added to Vero cells (do not express gD2) and their cytotoxicity was measured by addition of MTS reagent (Promega, Madison, WI). Triton X-100 was added at 0.05% to the first dilution to serve as a positive control for cytotoxicity, and it diluted as the other samples were. Dilutions of each protein were added to wells in triplicate and error bars represent standard deviation.

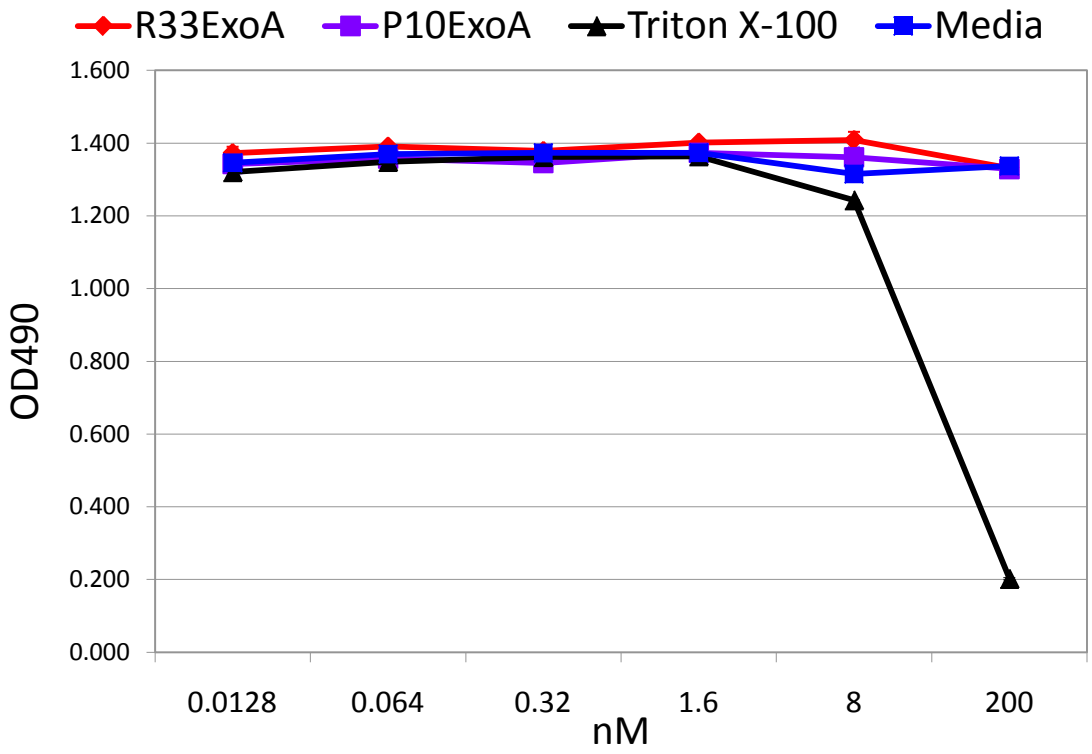


Figure 4.3.B: Toxicity of VHHExoA on Vero cells and z4/6 cells (express gD2)

B) Dilutions of VHH-ExoA proteins were added to z4/6 cells (express gD2) and their cytotoxicity was measured by addition of MTS reagent (Promega, Madison, WI). Triton X-100 was added at 0.05% to the first dilution to serve as a positive control for cytotoxicity, and it diluted as the other samples were. Dilutions of each protein were added to wells in triplicate and error bars represent standard deviation.

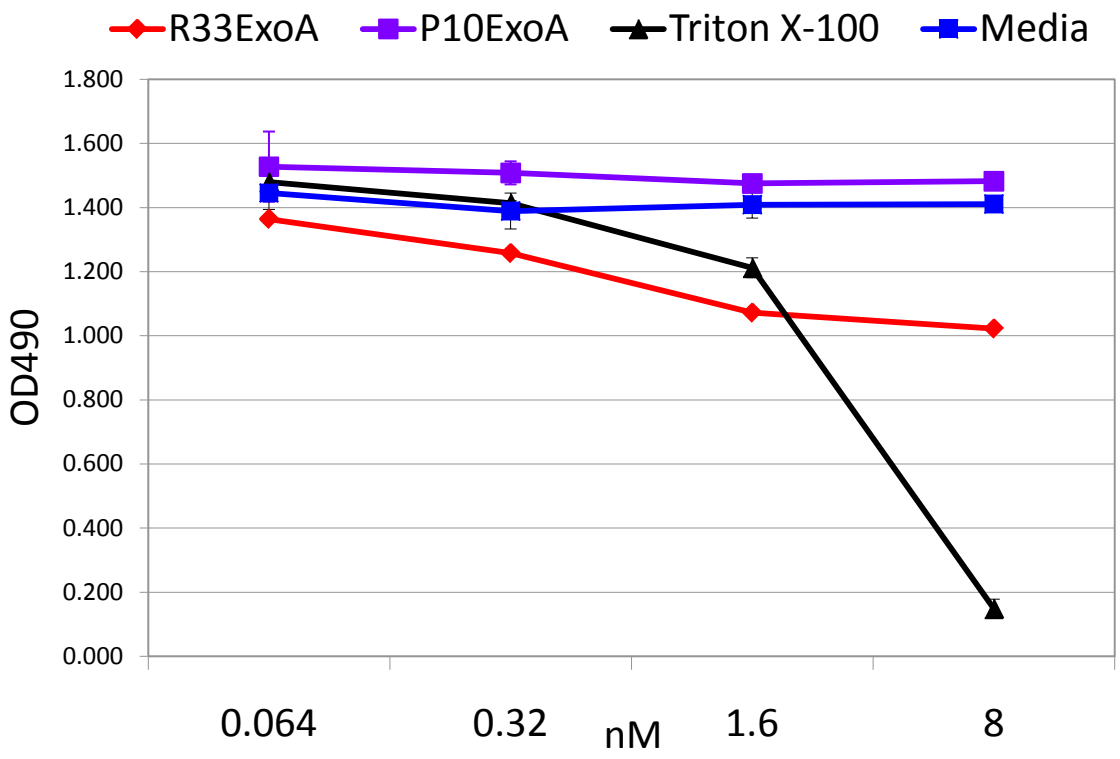


Figure 4.4: VHHExoA Infectious Center Assay

HSV-2 infected Vero cells were treated with dilutions of VHHExoA, R33, or PBS for about 16 hrs. Infected cells were then harvested and diluted in uninfected Vero cells to assay for the number of infectious centers that remain. This is a representative graph from four independent experiments. Error bars represent standard error of the mean.

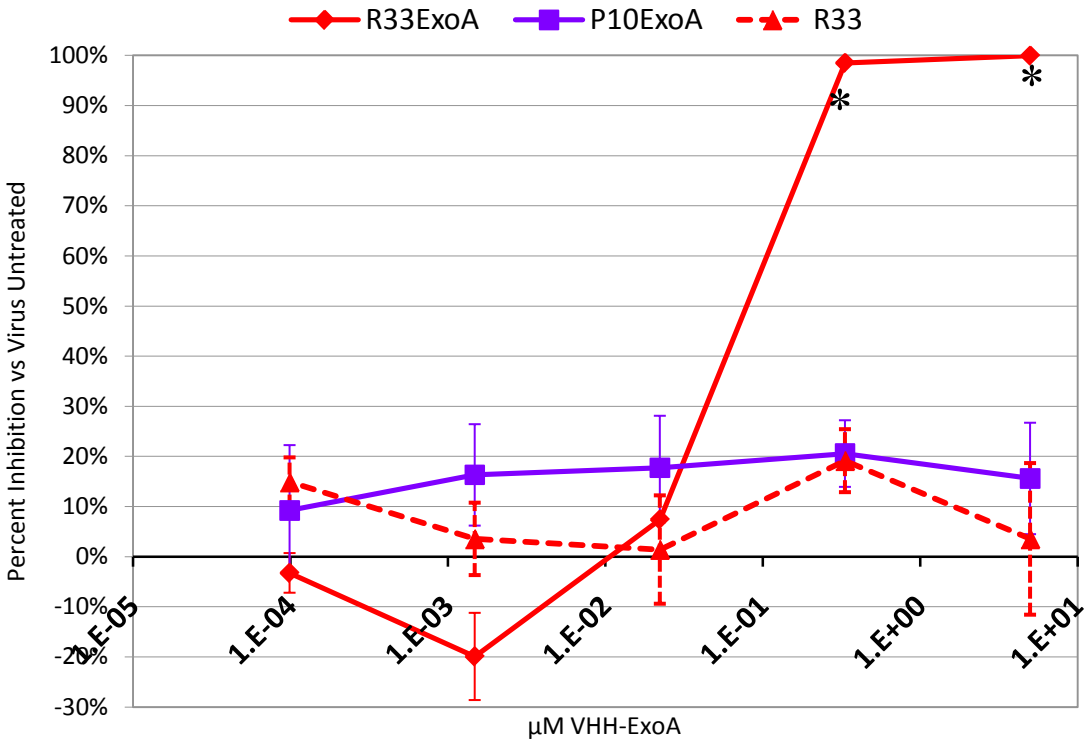


Table 4.1: Testing VHHExoA in Vaginal HSV-2 Animal Challenge Model

To test if VHHExoA immunotoxins can serve as an effective microbicide to prevent vaginal HSV-2 infection, Depo-provera treated mice were challenged with 10 ID_{50} virus inoculum mixed with PBS or VHHExoA. Mice were vaginally treated with additional doses of PBS or VHHExoA at 6, 24, and 48 hrs post-challenge. Three days after challenge, mice were lavaged to assay for presence of virus. Statistical significance was calculated by Fisher exact test (two-tailed).

Group	Viral Input	Treatment	# Infected	Total #	% Infected
Virus Only	10 ID_{50}	PBS	8	9	89%
R33ExoA	10 ID_{50}	10 uM	3	8	38%
P10ExoA	10 ID_{50}	10 uM	7	8	88%

Fisher exact test, two-tailed:

Virus Only compared to R33ExoA: $p = 0.0498$

P10ExoA compared to R33ExoA: $p = 0.1189$

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EDUCATION AND TRAINING

Ph.D., December 2013 (expected)
Molecular Microbiology and Immunology
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

Bachelor of Arts in Biology, 2007
Cum Laude
College of the Holy Cross, Worcester, MA

DISSERTATION

Geoghegan, E.M. 2013. Identification and Applications of Llama-Derived Single Domain Antibodies Binding to Glycoprotein D of Herpes Simplex Virus 2. Ph.D., Johns Hopkins Bloomberg School of Public Health, Baltimore, MD. Advisor: Richard B. Markham

HONORS AND AWARDS

Sommer Scholarship
2007-2013
Johns Hopkins Bloomberg School of Public Health

St. Ignatius Scholarship
2003-2007
College of the Holy Cross

PRESENTATIONS

Melissa A. Farrow, **Eileen M. Geoghegan**, Dimas C. Espinola, Shannon M. McKernan, and Ann M. Sheehy. (2007) "Comprehensive mutational analysis of APOBEC3G." Conference on Retroviral and Opportunistic Infections, Los Angeles.

RELEVANT SKILLS
Molecular biology: PCR, molecular cloning, protein expression and purification (bacterial, yeast, and baculovirus systems), ELISA, Western blotting, phage library assembly and biopanning, flow cytometry, immunohistochemistry

Animal experience (mouse and rat): vaginal lavage, intranasal and intravaginal immunizations, viral challenge, injections

Tissue culture: cell culture, viral neutralization assays, plaque assay

CERTIFICATES
Vaccine Science and Policy Certificate
2007-2009
Johns Hopkins Bloomberg School of Public Health

TEACHING
Public Health Perspectives
2010-2012
Teaching Assistant
Johns Hopkins Bloomberg School of Public Health

ACADEMIC SERVICE
Student Representative to Faculty
2009-2010
Molecular Microbiology and Immunology
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INTERNSHIPS
Regulatory Affairs Intern
Summer 2006
Wyeth Pharmaceuticals, Wilmington, MA

LEADERSHIP POSITIONS
Captain, 2013
US Women's National Team
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PROFESSIONAL ACTIVITIES
American Society of Microbiology, Member 2007-2013
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